THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. VII FEBRUARY, 1926 No. 1

ON SYSTEMIC FACTORS IN THE GENESIS OF CANCER.

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Received for publication November 5th, 1925.

THE paper deals with the problem of resistance to the genesis of cancer. In the past the term "resistance" has been used exclusively in relation to the transplantation of cancer. It was shown in 1907 by Bashford, Murray and Cramer that mice in which a transplanted tumour had disappeared either spontaneously or after treatment with radium were resistant to a subsequent transplantation. Further investigation showed that this resistance to transplantation is dependent partly at any rate to an interference with the process by which a tumour establishes itself after transplantation. It is doubtful whether there is in such animals any active interference with the growth of the transplanted cells. This phenomenon, however, has not necessarily any bearing on the question whether there is any resistance to the genesis of cancer.

The problem of resistance to the genesis of cancer is one of great importance, and it has acquired an even greater significance by the advance in our knowledge of the ætiology of cancer made by the recent work of Gye (1925). Gye's work may be interpreted in the sense that two factors are responsible for the local process transforming a normal cell into a cancerous cell—a living virus and a specific chemical substance. The introduction of a virus into the ætiology of cancer raises at once the question of the reaction of the whole organism to the presence of a virus, and thus suggests the possibility of systemic factors influencing the genesis of cancer. For the purpose of experimental investigation the question may be formulated in this way: Is the genesis of cancer dependent entirely on the local reaction of the cells subjected to chronic irritation, or are there in addition systemic factors which influence this reaction and in this way determine the onset of the disease? In the induction of cancer by tar-painting the tumour appears almost without exception in the painted area. In the large number of experiments of tar-painting in mice in this laboratory only three instances have occurred where a

tumour appeared outside the area actually painted. Even in these three exceptional cases the tumour appeared in the immediate neighbourhood of the painted area, and in two of these cases after an additional trauma had been inflicted, namely an incision of the skin around the painted area. It is probable that in these three instances either the process of chronic irritation induced by tar had spread a little beyond the painted area, or that the animals by scratching conveyed some of the tar to the neighbourhood of the painted area. This strict local limitation of the response to tar-painting has also been the general experience of almost all observers who have used this method. Considered by itself, the exclusively local response to tar would suggest *primâ facie* the explanation that cancerogenesis is exclusively a local process, in which only the cells subjected to chronic irritation are concerned without the intervention of general systemic factors.

Other facts, however, suggest the intervention of general systemic factors modifying and determining the local process of cancerogenesis. There are, for instance, great differences in the readiness with which in different individual mice cancer can be induced by tar-paintings applied under equal conditions. The routine method as practised at present in this laboratory is as follows: Gas coal tar is washed with water. The water is decanted, and the washed tar extracted either with ether or with chloroform. The ethereal or chloroform extract is decanted, and the solvent evaporated until a thick syrupy mass results. A small amount of ether or chloroform, as the case may be, is then added, so as to give the extract a fluid consistency suitable for painting. This extract is then applied twice weekly to the epilated skin of mice between the shoulder-blades, so that a very small area not more than 5 mm. in diameter is subjected to the effect of tar. With this routine method the first growths begin to appear as warts in a few animals in the fifth month of painting. During the sixth and seventh months they appear more frequently; in some animals they do not appear until the ninth month or even later, and some remain negative even after a year. There is, therefore, a very considerable difference between individual animals in their resistance to cancerogenesis by tar-painting.

Facts such as these suggest the possibility of the existence of general systemic factors which we may designate by the term "general resistance," but they do not conclusively prove it. The varying resistance might be due to differences between different areas of cells in the skin epithelium, and they have been so interpreted.

More conclusive evidence is furnished by the discovery of J. A. Murray (1923), that it is difficult to induce by tar-painting a carcinoma in mice which had previously developed a carcinoma either spontaneously or by tar-painting. Thus mice in which a spontaneous carcinoma of the mamma had developed and had been removed by operation are much more resistant to the induction of cancer by tar-painting than normal mice. The same holds good for mice in which a previous course of painting with tar had produced a carcinoma which had thus been removed by operation. Painting with tar applied to such animals frequently produces only an atrophic condition of the skin even after nine months of painting or more. If the second course of painting does induce a tumour a much longer period is required than was necessary for the

first tumour. I have been able to confirm these results in three mice in which a preliminary painting with tar had produced a carcinoma which had been removed by operation. Murray has pointed out that this fact explains the rarity of the occurrence in man of multiple tumours in different organs. Thus although the mamma and the uterus are frequently affected by cancer either in the one or in the other organ, it is rare to find cases in which both these organs are affected by cancer in the same individual. It seems difficult to interpret these findings otherwise than by assuming that the induction of the first cancer induces a general resistance to the induction of subsequent carcinomata.

Even this, however, does not entitle us to conclude that a general factor of resistance determines the onset of the first tumour. Maisin (1923) claims to have demonstrated the existence of such a general factor by experiments in which a preliminary painting of tar at one site, by itself supposed to be inadequate for carcinogenesis, produced a carcinoma at this site when it was followed by tar-painting at a second site.

From such experiments it has been concluded that tar-painting, in addition to its local effect, has a general effect, the absorbed tar producing an intoxication which diminishes the general resistance. Yamagiwa (1925) has criticized these and similar conclusions by Bayet, Bierich, Maisin and Lipschitz. Moreover, since Leitch (1922) had shown that an area of skin subjected to tar-painting over a certain period without visible effect, and then left alone, may after an interval of time develop tumours, and since it has been shown by Findlay (1925) that a single application of tar may lead under especially favourable conditions to the development of a carcinoma, Maisin's experiment is open to obvious objections that the preliminary cause of the tar-painting may have been adequate for cancerogenesis. In order to test the possibility that the absorption of tar in the course of tar-painting diminishes the general resistance of the animals, the following experiment has been carried out.

Mice were painted on the back of the neck with tar for six months, but only *once* weekly instead of *twice* weekly as in the routine method. The object was to allow of an absorption of tar over as long a period as possible without producing a tumour. The painted area was then widely excised. The neighbouring skin over the left shoulder was then painted with tar twice weekly. The excised skin was fixed in Zenker and examined microscopically. In no case was there any evidence of a carcinomatous change. The skin showed in most cases hyperplasic changes similar to those figured by Champy (1923) as the earliest effects of tar-paintings. If the preliminary treatment with tar over six months had diminished the resistance to cancerogenesis, the tar tumours should have appeared sooner in this batch than in a control batch of normal mice painted at the same time. The results expressed graphically in Fig. 1, in which each circle representing a mouse with a tar tumour is entered at the time when it first appeared, show that the appearance of the tar tumour had not been accelerated by the preliminary treatment with tar. In addition to a control batch of normal mice, another control was subjected to tar-painting in which without any preliminary tarring a piece of skin in the centre of the back had been removed, so as to test the possibility that the operative trauma had exercised any influence. In this second control batch

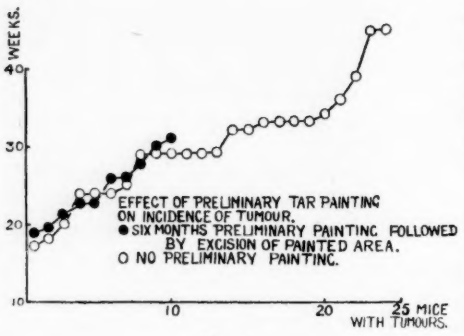

FIG 1.

the tumours appeared at the same time as in the first control batch, and as in the batch subjected to preliminary tarring. In the figure the two control batches are represented in the one curve labelled "no preliminary painting."

A similar experiment with similar results had been carried out by Dr. J. A. Murray. Although not yet published, Dr. Murray has kindly allowed me to refer to it briefly. In this experiment mice were painted twice weekly on the back of the neck. Then tarring at this site was discontinued, and a new site, the flank, was subjected to tarring twice weekly. The tumours at the second site appeared after the usual interval of time.

These experiments give no evidence that the absorption of tar affects the general condition of the animal in such a way as to influence the subsequent local reaction. The results are, however, merely negative, and do not exclude the existence of general systemic factors. Quite recently, after the present investigation had been concluded, Maisin and Masse (1925) have published more convincing evidence of the systemic effect of tar in reducing the general resistance to the genesis of cancer. They found that mice which received repeated subcutaneous injections of a drop of tar over four months, and were then painted with tar on the neck, developed tar cancers in the painted areas more rapidly and more frequently than normal control mice. They also state that in the injected mice the carcinomata grow more rapidly. Another investigation bearing on this point is that of Möller (1924), who found primary carcinomata of the lung in rats which had been subjected to tar-painting over a long period (a year or more), although no tumour had appeared in the painted area of the skin. Möller concludes that these lung carcinomata arose from the epithelium of the bronchi. But, as Möller himself points out, these results cannot be used as evidence in support of the view that there is a general resistance which has been broken down by painting with tar, since they can be explained readily as a local response of the epithelium of the bronchi either to inhaled particles, or to tar which has been absorbed and is excreted by the lungs. So far, then, the evidence for the existence in normal animals of systemic factors influencing the genesis of cancer is not very conclusive. This is due partly to the use of *one* agent—tar—to elicit the local reaction and to demonstrate the existence of systemic factors.

In order to test the matter further, it seemed desirable to influence the general condition by agents different from that used to elicit the local reaction. The possibility suggested itself that the absorption of the animal's own damaged cells might be a general factor determining the local reaction, since such a process is likely to occur in chronic irritation. To test this point the spleen was excised under ether anæsthesia in 63 mice. It was rapidly

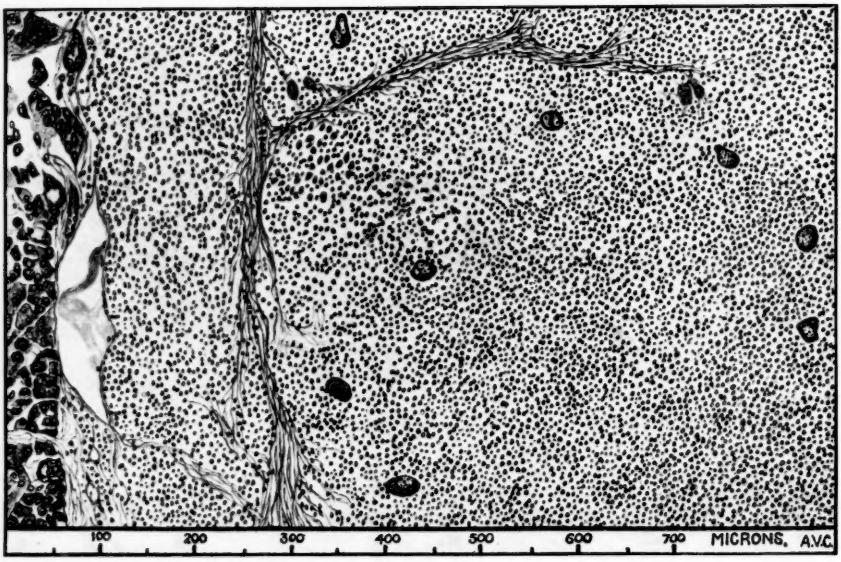

FIG. 3.—Spleen of mouse seven months after operation: fixed in Zenker's fluid: × 150. The regenerated spleen shows giant-cells and trabeculæ, and is adherent to the pancreas, as shown on the left of the figure.

minced with scissors and returned at once to the abdominal cavity through the opening in the abdominal wall, which was then closed with catgut stitches. After having recovered from the operation these animals were then painted with tar twice weekly. A control batch of 50 normal mice was painted at the same time. Painting began on 7 . 11 . 24. The results are given in Table I and graphically in Fig. 2. They show a very distinct acceleration in the appearance of tumours in the mice subjected to splenectomy and spleen autoplasty.

TABLE I.

Date.	Splenectomy. Number of negative mice alive.	Splenectomy. Number of mice with tar tumours dead and alive.	Normal. Number of negative mice alive.	Normal. Number of mice with tar tumours dead and alive.
7 . 11 . 24 .	63	—	50	—
5 . 3 . 25 .	48	6	30	—
8 . 5 . 25 .	25	23	21	2
3 . 7 . 25 .	10	37	9	8
1 . 9 . 25 .	3	41	5	10

For the sake of convenience the operated animals will be referred to briefly as "splenectomized mice." But it must be understood that in every one of these animals an extensive regeneration of the spleen had taken place. Sometimes the regenerated spleen attained the bulk of a normal spleen. This regeneration must have occurred from part of the introduced spleen-pulp, since no such regeneration has been observed by us to occur after splenectomy

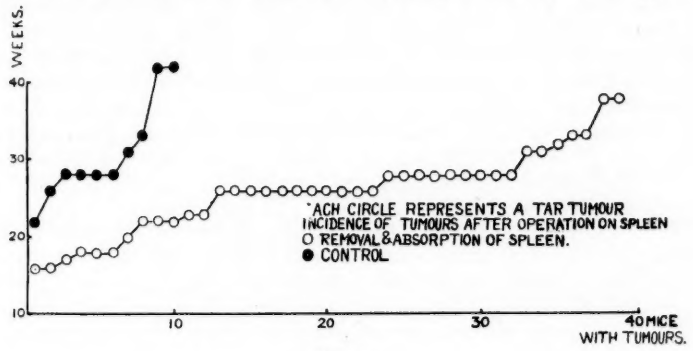

FIG. 2.

not followed by reintroduction of the spleen emulsion. It is curious that this regeneration always took place at the normal site of the spleen—namely the tail of the pancreas. In the majority of cases the regenerated spleen was adherent with one surface to the pancreas and with the other to the abdominal wound. The regenerated spleen had histologically the typical structure of spleen as shown in Fig. 3. This regeneration excludes the absence of spleen tissue as a possible factor in the acceleration of cancrogenesis.

Table I shows that a considerable number of tumours appeared in the fourth month. As stated above, tumours never appear in our normal mice before the fifth month of tar-painting if the routine method is used, and only very few appear in the fifth month. At the end of the sixth month there were nearly as many mice with tar tumours in the splenectomized mice as there were negative mice. The control batch gave only two tar tumours with 21 negative animals. The percentage of animals with tar tumours at the end of the experiment is not a fair criterion of the resistance in an experiment of this kind, because the more tarring has to be prolonged to produce a tumour the more likely are the animals to die as the result of the prolonged tarring. We find accordingly in the control batch a much higher mortality, which in part, at any rate, must be referred to the more prolonged tarring to which the majority of the animals had to be subjected. It is significant, however, that at the end of the experiment the control batch with fewer animals surviving had 5 negative animals, against only 3 negatives in a larger number of survivors in the splenectomized group.

Another significant fact is that in the operated animals 5 of the tar tumours had an exceptionally rapid growth.

Primâ facie the results would appear to confirm the supposition which the experiment was supposed to test that the absorption of the animal's cells predisposes to the genesis of cancer. The consideration that such a process probably occurs in chronic irritation lends support to the view that it is a factor concerned in the genesis of cancer. In this connection reference may also be made to an observation of Walton's (1915), which has not received the attention that it appears to deserve. In his experiments on *in vitro* cultivation of rabbits' tissues, he found that the injection of a rabbit's liver-cells into another rabbit produced in the plasma of this second rabbit a change to the effect that liver-cells grown in this plasma *in vitro* were stimulated to increased growth. The possibility can, however, not be excluded that in our experiments some accidental and unintentional factors were concerned in the effect—such as liberation of the virus, or its accidental introduction in the course of the operation.

CONCLUSIONS.

The results indicate that there are systemic factors influencing the genesis of cancer, and that the experimental procedure adopted in this experiment had diminished the resistance against the genesis of cancer. It is at present not possible to say with certainty whether this effect was due as intended to the absorption of damaged cells of the animal, or whether other accidental factors were concerned in it.

Incidentally, it was found that autotransplantation of the minced spleen tissue into a splenectomized mouse leads to regeneration of the spleen.

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THE DETERMINATION OF THE AMOUNT AND THE COMPOSITION OF THE FAT OF FÆCES. I. INVESTIGATION OF A "WET" METHOD AND COMPARISON WITH THE "DRY" METHOD.

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Received for publication November 22nd, 1925.

THE determination of the composition of the fat of fæces should, theoretically, offer useful information as to the efficiency of the digestive and absorptive processes. While such determinations are frequently made in the clinical investigation of cases of disease of the alimentary tract, their value has frequently been questioned. There is no doubt that the results obtained by the methods in general use are as a rule not worth the trouble taken to obtain them. Careful consideration of these methods, however, shows that they are subject to considerable inaccuracies, and suggests that therein lies the chief reason for the lack of value of the results obtained. The present investigation was therefore undertaken with the principal object of deciding ultimately how far results obtained by an improved method may be useful for clinical purposes.

All the present well-known methods of analysis involve the drying (by heat) and powdering of the fæces with subsequent extraction by a volatile fat solvent, usually ether. This is the principle of the method of Folin and Wentworth (1910), and of the modifications introduced by Hutchison (1920), Holt, Courtney and Fales (1919[1]), Cammidge (1914) and others.

It is well known, however, that most fats when in a finely divided state are readily hydrolyzed when heated in the presence of bases capable of combining with the fatty acids liberated by such hydrolysis, and to a less extent in the presence of acids. All these conditions are present in the

drying of fæces. Thus the composition of the fat of the dried specimen is likely to differ from that of the fresh wet specimen. To determine what this difference amounts to it is necessary to have a means of analyzing fæces in the fresh condition. Such a method was published by Saxon (1914), but appears to have received little attention in this country.

Holt, Courtney and Fales (1919[2]), in the study of the fæces of breast-fed infants, have noticed that there is a difference between the results of analysis of fresh and of dried specimens, but quote the analysis of only one specimen by both methods, and conclude that while drying does alter the relation between soap and free fatty acids, it causes no change in the values for neutral fat. In view of the great variability in the chief factors concerned, these conclusions seem to have been arrived at on insufficient data. I have therefore made a more complete investigation of Saxon's wet method and the dry method of Cammidge. The details of the latter are already well known. The procedure I have adopted for the former is as follows:

The sample is first thoroughly mixed, using a pestle and mortar if necessary to ensure uniformity. If liquid, a portion of the stool weighing about 5 gm. is poured into each of three stoppered weighing bottles, and the weight of each portion obtained. One weighing bottle is placed in a steam oven and dried to constant weight in order to determine the percentage of dry matter contained in the stool. Each of the remaining portions is washed into a 100 c.c. graduated glass-stoppered cylinder of uniform width throughout. To the contents of one cylinder 3 c.c. of concentrated hydrochloric acid are added, and then distilled water to 30 c.c. The contents of the second cylinder are diluted to 30 c.c. also, without the addition of any acid. If the stool is solid a quantity of about 10 gm. is placed in a weighed porcelain evaporating dish or crucible provided with a glass rod 2 in. to 3 in. in length, flattened at one end. Having obtained the combined weight of dish (and rod) and fæces, a quantity of the latter weighing 2 to 3 gm. is withdrawn on the flattened end of the rod and introduced into the cylinder by touching the cylinder wall with it below the ground portion at the mouth The dish and contents together with the rod, with any fæces still adhering to it, are then re-weighed. A second weighed quantity of fæces is similarly transferred to the second cylinder, after which the dish, rod and contents are dried to constant weight in the steam oven. Acid and distilled water are added to the cylinders as in the case of liquid stools. To each cylinder are now added 20 c.c. of ether, and the cylinders vigorously shaken for 5 minutes. Then, after standing for a few moments, 20 c.c. of 95 per cent. alcohol are added to the neutral cylinder and 17 c.c. to the acid cylinder. The alcohol is mixed with the other contents by giving the cylinders a sharp circular movement. Some rise of temperature occurs, and the cylinders are therefore stood in cold water in the sink for a short time, after which the contents are vigorously shaken for 5 minutes. The cylinders are then allowed to stand to allow the ether layer to separate. If separation does not occur readily gentle movement of the cylinder in a circular direction often helps, with the previous addition of a few drops of alcohol in most cases or ether in others. Experience will indicate the best means to adopt in any particular case, but once this has been obtained satisfactory separation need cause no difficulty.

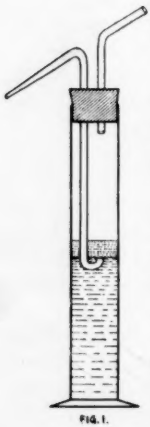

FIG. 1.

The upper layer is blown off into a fat extraction flask by converting the cylinder into a wash-bottle by the addition of a cork carrying glass tubes as shown in the sketch (Fig. 1). The exit tube is turned up at the lower end to avoid any disturbance of the separating surface by upward currents in the liquid. The removal of the ether layer is made complete by addition to and removal from the cylinder of three successive 5 c.c. quantities of ether.

A second extraction is then made by adding another 20 c.c. of ether to each cylinder, shaking for 5 minutes, separating and transferring the ether layer as before. From the combined extracts in the flask the solvent is evaporated, and the residue is dried and dissolved in petroleum ether. The solution is filtered into a weighed flask and the petroleum ether evaporated

off. After drying again the weight of extracted fat is obtained. Titration of the fat of the neutral extraction in benzene solution by N/10 sodium alcoholate completes the analysis. All results are calculated as a percentage of the dry matter of the fæces. They are thus strictly comparable with the results obtained by the dry method.

The purification of the extract with petroleum ether is necessary in this method, since ether in the presence of alcohol and water extracts material other than fat in sufficient quantity to affect the results considerably if not removed.

The result of the titration of the neutral extract gives the value for free fatty acids and for neutral fat. The latter value alone should theoretically be obtainable by titration of the acid extract also. In Saxon's method, however, the neutral fat value as obtained by this means from the acid extract is always considerably higher than the value obtained from the neutral extract.

The following example illustrates this:

Neutral fat by titration of acid extract, . 7·82 per cent. of dry fæces.
„ „ „ neutral „ . 5·78 „ „ „

One possible explanation is that during extraction some splitting of neutral fat takes place in the neutral extract, which does not take place, or takes place to a less extent, in the acid extract. If splitting is occurring, then the value of the method is entirely lost. It was therefore necessary to investigate the matter fully.

The number 284 as the molecular weight of the fatty acids, which is the basis of the calculation of these acids from the titration results, is an average one. To find out if any discrepancy between calculated and actual values for neutral fat existed, which might in some degree explain the problem in hand, the neutral fats from both acid and neutral extracts were isolated as follows:

The titrated fat in benzene solution was evaporated to dryness, and the residue of sodium soaps and neutral fat was extracted with petroleum ether. Evaporation of the petroleum ether extract gave the neutral fat from each of the original fæces extractions. The following is representative of a number of results obtained:

Neutral fat in acid extract, isolated	0·066 gm.
„ „ calculated from titration .	0·069 „
Neutral fat in neutral extract, isolated	0·047 „
„ „ calculated from titration	0·048 „

The agreement between actual and calculated figures is sufficiently close to show that the factor 284 used in the calculation is substantially correct.

It was ultimately shown that splitting during extraction does not occur by the following experiment:

One portion of a specimen of fæces was analysed as usual by Saxon's method. Of the remainder, two extractions were made as follows:

(*a*) The usual quantity was ground up with 2 c.c. of concentrated hydrochloric acid in a small glass mortar; 15 to 20 gm. of plaster-of-Paris were added—more than enough to combine with all the water

present. The whole was thoroughly ground up and left for 1 to 1½ hours to set, and was then re-ground.

(*b*) A similar quantity was ground up with plaster-of-Paris without the addition of any acid, and later re-ground.

Each of the ground mixtures was extracted in a Soxhlet apparatus for three hours with dry petroleum ether. The extract was evaporated to dryness, weighed, dissolved in benzene and titrated as usual.

By this means the stool was dried rapidly and without the application of heat, and was diluted with a considerable amount of dry neutral inert material and extracted in the absence of water. Under such circumstances no hydrolysis of the neutral fat could take place. The results were as follows:

	Saxon's method.	Plaster-of-Paris method.
Total fatty matter (acid extract) . .	11·32 per cent.	11·00 per cent.
Free fatty acids and neutral fat (neutral extract)	9·56 ,,	9·48 ,,
Fatty acids as soaps	1·76 ,,	1·52 ,,
Free fatty acids	3·78 ,,	3·99 ,,
Neutral fat by titration of neutral extract	5·78 ,,	5·49 ,,
Ditto by titration of acid extract . .	7·82 ,,	5·25 ,,

The general agreement between the results of the two methods is good. With regard to the particular problem under discussion, the outstanding facts are the agreement of the figures for neutral fat as obtained from both acid and neutral extract in the plaster-of-Paris method, and the similarity of these figures to the result obtained from the neutral extract of Saxon's method. It appears, therefore, that it is not the neutral fat of the neutral extract which is lower than the true value on account of hydrolysis, but that, for some reason, the neutral fat of the acid extract is higher than the true value. Some change takes place in the free fatty acids of the acid extract which results in a portion of them not being subject to neutralization by alkali.

Further light was thrown on the subject by the two following experiments:

(1) A stool was analysed in the usual way, but before evaporating the ethereal extract from the acid extraction it was transferred to a separating funnel and washed three times with distilled water to remove hydrochloric acid. The washed extract was then evaporated as usual.

Neutral fat by titration of neutral extract . .	7·70 per cent.
,, ,, acid extract . . .	7·68 ,,

The removal of hydrochloric acid before evaporation thus resulted in very close agreement between the two values.

(2) A second stool was analysed, and, whilst the acid extract was washed free of acid before evaporation, the neutral extract was acidified before evaporation by the addition of 2 c.c. of concentrated hydrochloric acid. It was then found that—

Neutral fat by titration of acid extract (washed) .	9·35 per cent.
,, ,, neutral extract (acidified)	13·18 ,,

It is clear, then, that the higher values for neutral fat are due to the presence of acid during the evaporation of the extract. It will be remembered that the extract is not simply an ethereal solution, but contains also a certain amount of alcohol. At a certain stage of the evaporation practically all the ether is removed, while the alcohol remains for the most part in the flask. There is then at this stage an alcoholic solution of fatty acid and sterols which is being heated in the presence of a mineral acid. These are just the conditions which are favourable to the occurrence of esterification, and it appears therefore that the apparent increase of neutral fat is due to the formation in this way of either sterol or ethyl esters of the fatty acids.

In this connection it was considered to be of sufficient interest to carry out estimations of the unsaponifiable matter (which consists mainly of sterols—cholesterol, coprosterol, etc.) contained in the neutral fat as extracted by Saxon's method. This was done by completely saponifying the neutral fat with alcoholic caustic soda solution and subsequently extracting the unsaponifiable matter with ether. The following are the results obtained, the percentage of dry matter in each stool also being given, as the results will be referred to later in another connection:

Dry matter %.	Total neutral fat (% of dry fæces).	Unsaponifiable matter (% of dry fæces).	Unsaponifiable matter as percentage of total neutral fat.
20·83	11·78	1·30	11·0
16·39	8·20	0·64	7·8
26·00	5·31	1·02	19·2
27·63	6·22	1·79	28·8
24·61	6·69	2·15	32·1
33·95	6·34	1·60	25·2
14·47	7·53	1·61	21·4
38·09	7·55	1·98	26·2
23·67	7·89	2·28	29·0
21·93	9·47	2·33	24·6
18·48	8·22	1·42	17·3
27·76	11·66	3·88	33·3
Range	.	0·64–3·88	7·8–33·3
Mean	.	1·83	23·0

Assuming the mean value for unsaponifiable matter, viz. 1·83, consisted entirely of cholesterol, this would only account for an increase of 1·35 in the neutral fat value as a result of the formation of cholesterol esters. The actual increase is greater than this, as shown by the figures I have already given. Hence it would appear that the esterification which occurs results in the formation of ethyl esters, mainly if not entirely.

Since the completion of this work, Harrison (1925) has published the results of observations which show that extraction of fat in the presence of alcohol and acid gives too high a value for neutral fat, which he also attributes to the formation of ethyl esters of the fatty acids.

The occurrence of this esterification has one important consequence:

Many workers believe that little or no significance is to be attached to the relative proportion of soaps and fatty acids. They confine their attention to the relation between the split and the unsplit fat. Were it not for this esterification the values for split and unsplit fat could be obtained from the acid extraction only, without modification, and much labour thereby saved. The occurrence of this complication, however, makes it necessary either to wash the acid extract free of acid before evaporating, or to make a double extraction as already described in all cases where more information than the total fat content is required. The plaster-of-Paris method is here shown to advantage, since no alteration of the neutral fat occurs during the acid extraction, and a single extraction without modification could therefore be employed to give the amounts of split and unsplit fat.

Having investigated Saxon's method, it is now possible to compare its results with those of the method of Cammidge.

The first specimen analysed by both methods gave the following results:

	Cammidge.	Saxon.
Total fatty matter . . .	15·01 per cent.	16·10 per cent.
Neutral fat and free fatty acids	5·60 ,,	11·91 ,,
Fatty acids and soaps . .	9·41 ,,	4·19 ,,
Free fatty acids . . .	3·98 ,,	5·87 ,,
Neutral fat	1·62 ,,	6·04 ,,

(The figures represent percentages of the dry matter of the fæces.)

This specimen was from an adult in whom no disturbance of fat digestion and absorption was suspected, and the results by Cammidge's method lie within the limits he gives for normal persons. Two results of the analysis of fæces from pathological cases are as follows:

	Cammidge.	Saxon.
(1) Total fatty matter . . .	50·51 per cent.	56·32 per cent.
Neutral fat and free fatty acids	21·23 ,,	20·32 ,,
Fatty acids and soaps . .	29·28 ,,	36·00 ,,
Free fatty acids . . .	16·32 ,,	13·52 ,,
Neutral fat	4·91 ,,	6·80 ,,
(2) Total fatty matter . . .	26·35 ,,	30·93 ,,
Neutral fat and free fatty acids	20·46 ,,	30·93 ,,
Fatty acids and soaps . .	5·89 ,,	*Nil.*
Free fatty acids . . .	16·53 ,,	23·74 ,,
Neutral fat	3·93 ,,	7·19 ,,

Many more cases might be quoted, but these three are sufficient to illustrate the differences in results obtained by two methods. They also show that whatever factors cause these differences do not operate to the same extent in all cases. The outstanding features are:

(1) The total fat as determined by the wet method is higher than when determined by Cammidge's method, *i.e.* the wet method affords a more efficient means of extraction. In many cases the difference is negligible for clinical purposes, but this is not always so.

(2) In general there is a transference of fatty acids from the free to the combined state by Cammidge's method as compared with Saxon's, but this is not always the case.

(3) The neutral fat by Cammidge's method is less than by Saxon's method, and here again the relation between the results by the two methods is very variable.

One cause of these differences has already been suggested, namely that significant chemical changes occur during the drying process. Another explanation which would equally account for the findings is that there is very considerable inefficiency in the neutral extraction by the dry method. This would result in altered values for soaps and free fatty acids, and lower values for neutral fat. The following experiment was undertaken to show if one or both causes are in operation:

A number of specimens of fæces were divided into two portions. The first portion was analysed at once by Saxon's method. The second portion was dried in the steam oven and then divided into two parts, of which the first was subjected to neutral extraction by Cammidge's method, and the second was treated with water and subjected to the wet neutral extraction by Saxon's method. The value for the total fat was taken from the acid extraction of the first portion. I have chosen the following results from a much larger number to illustrate important points:

No. of specimen.	Method.	Total fat.	Total neutral extract.	Soap fat.	Free fatty acids.	Neutral fat.
1	Wet	27·57	18·40	9·17	6·62	11·78
	Wet after drying	„	18·30	9·07	7·51	10·79
	Dry	„	15·74	11·83	5·41	10·33
2	Wet	12·50	11·75	0·75	5·06	6·69
	Wet after drying	„	11·00	1·50	4·66	6·34
	Dry		8·05	4·45	4·16	3·89
3	Wet	15·54	13·27	2·27	5·05	8·22
	Wet after drying	„	12·00	3·54	3·41	8·59
	Dry	„	9·15	6·39	8·59	6·14
4	Wet	24·94	15·25	9·69	8·12	7·13
	Wet after drying	„	18·80	6·14	14·37	4·43
	Dry	„	11·71	13·23	8·73	2·98

The actual effect of drying is seen in a comparison of the results of the neutral extraction by Saxon's method before and after drying. It will be noted that the amount of hydrolysis of neutral fat which occurs is variable. In some cases it is negligible, and this would explain why those who have made comparisons of the results of two or three analyses only have decided that hydrolysis does not occur. In other cases, however, the hydrolysis is considerable, and cannot be overlooked. There is also a disturbance of the original relationship between soaps and free fatty acids, not always in the same direction. Moreover, I have noticed that the effect of drying depends on the time and temperature of the process. The quick drying of a small portion of a specimen in the steam oven causes less change in general than by

drying the whole of the specimen slowly on the water-bath. Further, the quickly dried specimen appears to be in a mechanical condition which is more favourable for extraction than the slowly dried one. These factors probably explain why, with rapidly dried specimens, I have frequently obtained from undoubtedly normal stools much higher figures for neutral fats (*e. g.* specimens 2, 3 and 4 above) than are quoted by Cammidge.

A comparison of the two different methods of extraction of the dried fæces shows that Cammidge's method is not sufficiently efficient.

It is therefore obvious that any method of analysis of dried specimens of fæces will show a fat composition, which varies to a greater or less degree from that of the specimen as passed. What is of importance is that, not only are the dry methods inaccurate in a purely scientific sense, but the inaccuracies are such as to lead to erroneous deductions with regard to the clinical significance of the figures obtained.

A significant comment on Cammidge's figures for the fæces of the normal adult is given by the figures I have quoted for the unsaponifiable matter alone. All these figures were obtained from fæces of normal adults, and it is seen that they are somewhat greater than Cammidge's figures for the whole of the neutral fat.

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THE DETERMINATION OF THE AMOUNT AND THE COMPOSITION OF THE FAT OF FÆCES. II. THE COMPOSITION OF THE FAT OF THE FÆCES OF THE NORMAL ADULT, AS ASCERTAINED BY THE "WET" METHOD, TOGETHER WITH SOME RESULTS IN CERTAIN PATHOLOGICAL CONDITIONS.

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Received for publication November 22nd, 1925.

In the previous paper it has been shown that the analysis of the fat of fæces after previous drying of the specimen gives results which are quite inaccurate, and which may be entirely misleading. In order to use the wet method therein described for clinical purposes, it is necessary to know what results are obtained by this method from normal fæces. I have therefore analysed the fæces of eighty adults, forty being men and forty women. These were hospital patients on the "ordinary" hospital diet, who, so far as could be determined, were not suffering from disorder of the alimentary tract. They were all surgical cases suffering from fractures, varicose veins, etc. Such cases represent a more useful standard for clinical work than healthy active individuals for obvious reasons. In deciding whether a sick person is or is not suffering from alimentary disease, the most useful standard will be the sick person who is not suffering from such diseases rather than the healthy active person.

The stools were analysed as soon as possible after being passed by the patient and uniform conditions and apparatus were used throughout.

The results are given in Table I, the figures for total fat, soap fat, free fatty acids and neutral fat being expressed as percentages of the dry matter present in the fæces. The percentage of dry matter which the fresh fæces contain and the percentage which the neutral fat forms of the total fat present are also given.

TABLE I.—*Men.*

Patient. No.	Age.	Fæces. Dry matter (% of fresh fæces).	Total fat.	Soap fat.	Free fatty acids.	Neutral fat.	Neutral fat as % of total fat.
1 (*a*) T. M—	24	18·52	11·86	2·10	4·71	5·05	42·6
(*b*) ——	—	19·27	20·20	7·75	3·37	9·08	44·9
(*c*) ——	—	26·60	18·98	8·06	6·20	4·72	24·9
2 (*a*) A. C—	75	19·89	21·46	7·39	3·80	10·27	47·8
(*b*) ——	—	20·43	23·17	6·99	6·83	9·35	40·4
3 W. K—	29	18·78	14·32	3·61	4·68	6·03	42·1
4 E. A—	17	12·32	7·28	2·09	2·70	2·49	34·2
5 J. B—	38	31·06	24·72	9·40	6·65	8·67	35·1
6 B. W—	53	24·84	13·45	2·17	5·10	6·18	46·0
7 F. F—	32	7·87	17·57	3·46	7·89	6·22	35·4
8 W. D—	19	13·62	21·19	5·99	4·98	10·22	48·2
9 R. H—	33	7·77	10·46	2·44	2·66	5·36	51·2
10 J. P—	55	22·20	17·98	2·65	7·19	8·14	45·3
11 W. W—	51	22·42	21·62	11·45	3·57	6·60	30·5
12 L. W—	36	17·32	11·32	1·76	3·78	5·78	51·0
13 R. T—	36	26·87	23·37	3·85	7·72	11·80	47·4
14 R. K—	16	17·12	15·54	4·62	3·22	7·70	49·6
15 F. P—	38	24·91	14·86	3·85	5·44	5·57	37·5
16 W. ?—	68	10·46	11·84	1·47	5·00	5·37	45·3
17 G. B—	31	23·37	19·45	5·14	7·39	6·92	35·6
18 C. S—	52	6·31	16·86	3·18	5·18	8·50	50·4
19 R. C—	50	23·65	22·71	4·47	6·39	11·85	52·2
20 J. W—	40	17·25	15·62	2·46	6·93	6·23	39·9
21 T. B—	18	32·11	22·42	5·43	8·84	8·15	36·4
22 C. D—	18	27·26	18·73	5·96	5·88	6·89	36·8
23 E. C—	53	23·91	17·41	5·30	4·24	7·87	45·2
24 R. T—	32	26·45	22·20	7·81	6·10	8·29	37·5
25 T. D—	44	19·75	19·92	7·45	4·62	7·85	39·4
26 R. T—	36	20·67	16·12	3·55	5·88	6·69	41·5
27 T. B—	18	24·61	14·67	2·47	5·20	7·00	47·7
28 H. P—	26	25·94	21·20	8·08	4·89	8·23	38·8
29 E. W—	15	22·71	10·05	0·54	4·41	5·10	50·8
30 J. B—	38	28·47	24·76	9·49	5·89	9·38	37·9
31 E. B—	33	29·33	18·75	6·57	4·61	7·57	40·4
32 W. S—	65	22·25	20·33	6·81	6·22	7·30	35·9
33 T. D—	44	17·03	19·07	2·40	6·01	10·66	55·9
34 T. B—	18	9·23	15·47	4·81	4·24	6·42	41·5
35 H. S—	35	7·23	17·23	5·81	5·70	5·72	33·2
36 J. G—	35	20·89	15·93	2·48	7·75	5·70	35·8
37 D. H—	48	15·71	16·34	5·77	5·07	5·50	33·7
38 G. C—	25	25·01	14·04	1·20	8·08	5·36	36·6
39 G. R—	44	16·38	9·35	1·04	3·98	4·33	46·3
40 ? W—	50	24·20	18·44	2·52	10·00	5·92	32·2

Women.

Patient. No.		Age.	Fæces. Dry matter (% of fresh fæces).	Total fat.	Soap fat.	Free fatty acids.	Neutral fat.	Neutral fat as % of total fat.
1	(*a*) M. B—	70	25·54	19·87	4·95	4·99	9·93	50·0
	(*b*) ——	—	17·29	17·29	8·77	1·05	7·47	43·2
2	A. K—	57	25·17	20·53	0·93	9·29	10·31	50·2
3	? J—	42	22·10	16·10	4·19	5·87	6·04	37·5
4	E. S—	63	23·45	16·59	3·34	4·70	8·55	51·5
5	E. H—	27	17·02	20·93	6·38	6·75	7·80	37·3
6	E. F—	65	30·54	25·57	10·28	8·84	6·45	25·2
7	H. P—	78	13·73	12·62	3·11	3·47	6·04	47·8
8	R. B—	21	26·54	18·82	3·89	5·68	9·25	49·1
9	P. A—	20	31·18	18·15	2·83	6·82	8·67	47·8
10	E. C—	34	14·73	14·93	1·13	5·88	7·92	53·0
11	E. G—	49	17·62	18·27	5·30	5·30	7·67	42·0
12	M. H—	53	10·98	16·51	3·49	3·24	9·78	59·2
13	E. C—	34	11·82	10·43	2·52	4·21	3·70	35·5
14	A. B—	20	28·21	16·00	3·46	5·34	7·20	45·0
15	W. B—	18	17·24	13·61	1·82	3·61	8·18	60·1
16	A. F—	12	22·05	22·52	8·82	6·32	7·38	32·8
17	H. M—	16	23·15	16·18	6·42	4·47	5·29	32·7
18	M. R—	26	23·07	23·91	6·43	6·14	11·34	47·4
19	N. G—	23	33·81	14·09	1·04	5·50	7·55	53·6
20	M. M—	19	20·04	15·19	4·53	6·92	3·74	24·6
21	A. T—	53	17·65	15·87	3·13	8·51	4·23	26·7
22	K. M—	18	17·40	24·61	8·42	5·79	10·40	42·2
23	M. G—	21	32·19	11·77	0·62	5·49	5·66	48·1
24	E. C—	31	17·20	13·81	1·31	6·62	5·88	42·6
25	N. G—	33	26·00	11·29	1·68	4·30	5·31	47·0
26	A. F—	13	23·43	20·49	9·18	4·25	7·06	35·3
27	E. C—	34	19·98	12·39	0·97	4·94	6·48	53·5
28	M. R—	26	21·86	14·18	2·14	7·18	4·86	34·3
29	M. G—	40	20·37	20·61	8·03	5·57	7·01	34·0
30	K. M—	18	20·83	27·57	9·17	6·62	11·78	42·7
31	E. B—	38	16·39	22·22	7·46	6·56	8·20	36·9
32	J. S—	53	4·58	21·17	5·49	9·82	5·86	27·7
33	N. G—	23	21·29	20·78	5·14	4·96	10·68	51·4
34	E. B—	32	27·63	19·81	7·79	5·80	6·22	31·4
35	M. M—	29	24·61	12·50	0·75	5·06	6·59	53·5
36	M. R—	26	33·95	20·58	9·26	4·98	6·34	30·8
37	V. R—	15	23·02	16·30	3·33	5·88	7·09	43·5
38	H. S—	20	38·09	14·91	2·66	4·70	7·55	50·6
39	? P—	62	21·93	20·05	2·57	8·01	9·47	47·2
40	M. R—	38	18·48	15·54	2·27	5·05	8·22	52·9

The mean of the above figures is as follows:

	Dry matter.	Total fat.	Soap fat.	Free fatty acids.	Neutral fat.	Neutral fat as % of total fat.
Men (43 specimens) . .	20·3	17·4	4·69	5·56	7·16	41·4
Women (41 specimens) . .	22·0	17·7	4·51	5·72	7·45	42·9
Total (84 specimens) . .	21·1	17·5	4·60	5·64	7·31	42·0

and the range is:

4·6–38	7·3–27·6	0·54–11·4	1·05–10	2·49–11·8	24·6–60·1

For purposes of comparison the figures given by Cammidge are as follows:

	Total fat.	Soap fat.	Free fatty acids.	Neutral fat.
Mean (10 cases) . .	21·0	11·0	9·0	1·0
Range	8·5–25·2	4·8–16·0	8·0–13·0	0·8–2·0

The significance of the results by the wet method as shown in the table may now be discussed with respect to the various constituents which have been determined.

Dry Matter.

The amount of dry matter—and therefore of water—is extremely variable even in the normal stool. So great is the variation that the figures have in themselves no significance. They have been included in order to indicate the type of specimen whose fat analysis is given, *i. e.* whether the stool is a "wet" or a "dry" one, and also to ascertain the average amount of dry matter present in normal stools in order to obtain some criterion of what is a "wet" or a "dry" stool.

It will be seen that the average value for dry matter is 20–21 per cent. Stools with less than 15 per cent. are semi-solid or liquid, while those containing more than 26 per cent. are very firm. A comparison of the fat figures for stools with less than 15 per cent. of dry matter with those containing more than 26 per cent. reveals little difference in the type of result. I have obtained the average of the results in these two classes of stools and the figures are of considerable interest. They are:

	Total fat.	Soap fat.	Free fatty acids.	Neutral fat.	Neutral fat as % of total fat.
"Wet" stools, *i. e.* less than 15 per cent. dry matter (13 samples) . . .	14·9	3·46	5·00	6·44	43·1
"Dry" stools, *i. e.* more than 26 per cent. dry matter (18 samples) . . .	19·1	5·55	6·08	7·47	39·1

These two sets of figures agree well with the average for the whole of the eighty-four specimens already quoted. They show therefore that fat-splitting and fat-absorption have proceeded satisfactorily, and hence, in this respect, both series may truly be considered normal. The difference in water-content is simply due to the dry stools having remained for a longer time in the large intestine than have the moist stools, and the differences between the two sets of figures are due to this fact. The possible changes occurring in the large intestine have been referred to as unknown factors possibly affecting to some extent the value of fæces analyses of the type I have dealt with. In the light of these figures it would appear that the changes occurring are not of any great extent. While the figures for the total fat differ somewhat, the relations of the component parts to one another in the two series are very similar, and no difference in clinical significance is suggested by the figures. Hence it is probable that the result of the analysis is not a very incorrect picture of the composition of the fæcal fat at the end of the digestive and absorptive processes.

Nevertheless such differences as exist are interesting. The higher value for total fat in the fæces which have remained for a longer time in the large intestine gives support to the view put forward by Fleming and Hutchison

(1924), Hill and Bloor (1922) and others, that a fat excretion takes place in the large intestine. Of the increase, 50 per cent. is as soap fats, and this would explain how demineralization might occur if fat excretion becomes excessive. So far as the increase in the neutral fat value is concerned, a reference to the table of results in the previous paper showing the amount of unsaponifiable matter present in the fæces suggests that the drier fæces contain a greater proportion of sterols, etc., though the figures are not sufficiently numerous or definite to permit one to draw conclusions on this point.

Total Fat.

The average amount of total fat is decidedly less than that given by Cammidge's figures. This may be due to the larger number of specimens which I have examined. The upper limit is somewhat higher, but is closely comparable with that of Cammidge when the greater efficiency of extraction of the wet method is taken into account.

Free and Combined Fatty Acids.

The average of these values by the wet method shows a preponderance of free over combined fatty acids, but individual results vary so widely in this respect that it is difficult to understand the significance of the relationship between the two, or the value for clinical purposes of differentiating between the two, at any rate so far as adults are concerned.

Neutral Fat.

The difference between the value obtained for this constituent by the wet method and the value given by Cammidge is very striking. While the figures vary considerably among themselves, it is interesting to note that 80 per cent. of them are below 9. The percentage which the neutral fat forms of the total fat is probably a much more important value, and exhibits less variation. In 80 per cent. of the cases this is below 50. The differences between extreme values are somewhat large, but it will be seen that a larger number of the specimens give results which approximate fairly closely to the average.

After due consideration of the results the following may be suggested as a guide in the clinical use of the method :

(1) Any specimen in which the total fat amounts to more than 28 per cent. of the total dry matter is probably abnormal.

(2) Any specimen in which the neutral fat exceeds 12 per cent. of the total dry matter or 60 per cent. of the total fat should be suspected of showing evidence of deficient fat-splitting.

(3) Any specimen in which the total of split fat (*i.e.* free and combined fatty acids) exceeds 20 per cent. of the dry matter or 75 per cent. of the total fat should be suspected of showing evidence of deficient fat-absorption.

The stools of children from the age of about two years upwards are similar to those of adults as regards fat content and composition, and the figures obtained lie within the limits given for adults.

I have not as yet made a study of the fæces of infants by this method.

SOME RESULTS OBTAINED FROM ANALYSIS OF THE FÆCES BY THE WET METHOD IN CERTAIN PATHOLOGICAL CONDITIONS.

The analysis of the fat of fæces cannot of itself lead to a precise diagnosis. Theoretically, it should, however, serve to indicate whether fat-splitting or fat-absorption or both are impaired. In practice such analysis as a clinical procedure in the investigation of alimentary diseases has proved disappointing where the dry method of analysis has been used. Though the wet method has the advantage of giving a more accurate picture of the composition of the fæcal fat than the dry method, its true value as an aid to diagnosis can only be ascertained as a result of a comprehensive survey of the results obtained in each of several pathological conditions. Such a survey I have not yet completed, but the results of analysis which I have made of certain specimens may be given as indicating the type of results obtained in some conditions and the way in which they vary from the normal.

(1) *Catarrhal conditions of the small intestine.*—Amongst the specimens obtained from the eighty patients whose stools were examined in investigating the normal, two specimens differed markedly from the rest. and were to the naked eye abnormal, being wet, pale and frothy. The results of analysis are as follows:

	Total fat.	Soap fat.	Free fatty acid.	Neutral fat.
1. T. C—	47·94	16·96	18·02	12·96
2. A. K—	47·78	17·94	17·38	12·46

The condition was temporary in both cases, the normal figures of 2*a* and 2*b* (men) of the table being obtained for stools obtained one week before and one week after the stool giving the first of the above results, and 2 (women) of the table being obtained a fortnight after the second. The results are representative of a temporary catarrh of the intestine—the common diarrhœa—and may be contrasted with the wet stool which gives normal values. They show a considerable deficiency in fat-absorption with some deficiency of fat-splitting also.

The following shows the result obtained in a more chronic type of intestinal catarrh, the figures indicating some deficiency of fat-absorption with perfectly satisfactory fat-splitting:

Total fat	26·50
Soap fat	13·90
Free fatty acids	10·90
Neutral fat	1·70

Much mucus was present.

(2) *Obstructive jaundice.*—The average of four cases due to gall-stones was as follows:

Total fat	50·9
Soap fat	26·8
Free fatty acids	18·1
Neutral fat	6·0

Each case agreed closely with this average, which indicates considerable lack of absorption, with a normal figure for neutral fat.

(3) *Acute pancreatitis.*—The necessity for immediate operation in such cases does not allow of the opportunity for pre-operative laboratory investigation. In two cases from which fæces were obtained two days after operation the results were similar to those for obstructive jaundice. Gall-stones were found at operation in each case. The results were:

	(1)	(2)
Total fat	54·28	46·54
Soap fat	35·23	18·69
Free fatty acid	13·26	19·88
Neutral fat	5·79	7·97

Eight days after operation a stool from the second patient gave a result which was within the normal limits. The patient has made a satisfactory recovery.

In one case of subacute pancreatitis the fæces a few days after operation contained the following:

Total fat	29·50
Soap fat	7·50
Free fatty acid	9·94
Neutral fat	12·06

The figures suggest a slight degree of pancreatic insufficiency.

(4) *Subacute liver atrophy and cancer of the head of the pancreas.*—One case of each of these conditions has been investigated. Both results were typical of lack of fat-absorption, from deficiency of bile in the intestine, agreeing closely with the type of result for obstructive jaundice.

(5) *Tuberculosis of mesenteric glands.*—The figures obtained in two cases of extensive disease of this type in children gave the picture of considerable lack of fat-absorption, with, in one case, evidence of some deficiency in fat splitting.

(6) *Cœliac disease.*—One case of this disease in a boy, aged 12 years, showed:

Total fat	44·40
Soap fat	16·55
Free fatty acid	18·97
Neutral fat	8·88

The results obtained so far are seen, therefore, to differ appreciably from the normal, which has been ascertained, and to conform to certain types according to which function is impaired. They have agreed in this respect with clinical findings, supplemented in some cases by operation or post-mortem confirmation, and offer some hope that the method may prove of considerable value in clinical work. How far this hope is justified will be evident from the results of the further work which is now in progress.

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INSULIN AND GLUCOSE UTILIZATION: EFFECTS OF ANÆSTHETICS AND PITUITRIN.

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Received for publication January 16th, 1926.

It has been shown by Banting, Best, Collip, Macleod and Noble (1922) that the injection of insulin into rabbits results in a fall in the percentage of blood-sugar, followed by the production of hypoglycæmic convulsions, and this observation has been universally confirmed. Mann and Magath (1923) have likewise found that a rapid fall in the blood-sugar and the appearance of hypoglycæmic convulsions can be brought about by the excision of the liver in dogs. This naturally led one to inquire whether there was any similarity in the mechanism by which the hypoglycæmia was brought about in the two cases; whether, for example, insulin acted by preventing the liver from liberating glucose into the circulation. Comparison of the blood-sugar curves obtained after the administration of insulin to the intact animal with those from the decerebrate eviscerated preparation, after exclusion of the liver from the circulation by ligation of the portal vessels, revealed the fact that the rapidity of the fall in blood-sugar brought about by these two methods is comparable. Figs. 1 and 2 illustrate the findings in the case of the cat. In spite of the apparent similarity between these curves, it was necessary to consider other methods besides the cutting off of the supply of glucose from the liver, by which the removal of blood-sugar under insulin might be effected. Thus the rate at which the muscles and other tissues remove glucose

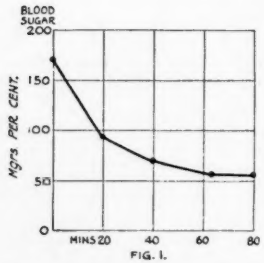


FIG. 1.

Cat; intact animal. Insulin, 10 units administered subcutaneonsly.

All the figures represent blood-sugar curves in the cat, the ordinates referring to the concentration of blood-sugar in milligrammes per cent. and the abscissæ to time in minutes.

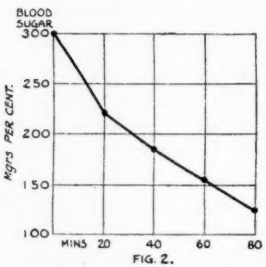


FIG. 2.

Cat, decerebrated and eviscerated; portal vessels ligated.

from the blood might be accelerated, or perhaps some inhibitory influence exerted through the liver upon the glucose utilization in muscle might be removed by insulin.

Regarding these various possibilities some information is now available. Thus, it has been shown by Dudley and Marrian (1923) that in mice which have been poisoned by large doses of insulin, the liver and muscles become depleted of glycogen. This, however, may have been the result of convulsions.

On the other hand, experiments by Macleod and Noble indicate that insulin antagonizes the glycogenolysis which occurs under the influence of adrenalin, while Cori, Cori and Goltz (1923), who examined the blood entering and leaving the liver, did not find evidence of increased output of glucose after small doses of insulin. Again, Hepburn and Latchford (1922) have shown that in the case of the isolated heart an increased removal of glucose takes place after insulin is added to the perfusion fluid, and this has been confirmed by Dale and Burn (1924).

Experiments by Cori, Cori and Goltz on rabbits, and by Lawrence on the human subject, in which arterial and venous blood were compared with respect to their content of sugar, indicate that an increased loss of sugar takes place in the tissues after the administration of insulin. In these experiments, since the liver was intact, the possibility remained that some action in the liver might have been necessary before the increased removal of glucose could be effected by the other tissues. Mann and Magath, however, working with dogs, found that when the liver was completely excised the average rate of fall after insulin was greater than in the controls. In an attempt to confirm this, experiments of a similar character have been performed upon decerebrate dogs, cats and rabbits in which the animals were eviscerated and the portal and renal vessels ligated. It was not possible, however, to draw any conclusions, since the curves obtained with insulin fell within the limits of variation in the controls. Again, owing to the steepness of the curve, it was difficult to show any definite increase in the rate of fall when insulin was thrown in after a preliminary period without it. In order to overcome this difficulty, the method of Dale and Burn was adopted in the experiments to be presently described, namely to inject glucose at a uniform rate which was sufficient to balance the fall due to exclusion of the liver, and in this way to bring out any changes arising from the injection of insulin. The results show that an increased removal of sugar takes place in the muscles under insulin, even when the liver is excluded. In this paper the findings of Dale and Burn are confirmed, while an attempt is made to measure the changes in the rate of removal before and after insulin and to investigate the influence of anæsthetics and of pituitrin. The effect of including the liver is also studied.

TECHNIQUE.

Cats were decerebrated by Sherrington's method, after brief etherization, and put on a heated operating table—the heat being regulated by means of electric lights placed beneath the copper table top. The temperature of the animal, taken in the axilla, was kept practically constant. Artificial respiration was begun immediately after decerebration, and was continued throughout the

experiment. Cannulæ were placed in the carotid on one side and in both jugular veins. The abdomen was opened, and, after ligation of both superior and inferior mesenteric arteries, the large and small intestines from the beginning of the jejunum downwards were removed. The œsophagus was then divided between ligatures, the cœliac axis and the portal vessels, together with the bile-duct, were tied, and the stomach, spleen and pancreas were removed. The renal vessels were then ligated, after which the abdomen was closed and the whole animal was wrapped up in thick layers of cotton-wool, while a warming lamp was placed over the abdomen. Transfusion of glucose was now begun. A special device was employed for this purpose, the object being to obtain a slow rate of injection at a uniform speed. It consists (Fig. 2A) of a screw, square on cross-section, held by a bracket and working

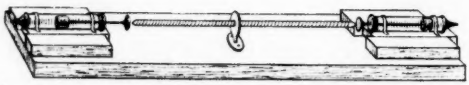


FIG. 2A.

on a wheel driven by a motor, a reducing gear being interposed so as to obtain a slow rotation. The screw in advancing was made to drive forward the piston of a 10 c.c. Record syringe, placed in line with it, and fixed, by means of clips, on a wedge of wood, which could be made to slide along so as to bring the syringe to any convenient distance from the screw. Another syringe was placed in a similar position at the opposite end of the screw, so that a second injection could, if necessary, be given, with but momentary interruption of the flow, by merely reversing the rotation of the screw. The syringe was connected by pressure-tubing with a cannula placed in the jugular. The rate of injection was regulated in such a way that the volume delivered by the syringe was only 5 c.c. per hour. In order to obtain a given rate of glucose transfusion reckoned as so many grammes of glucose per kilo of animal weight per hour, it was merely necessary to make up a solution of glucose of the requisite strength in Ringer's solution, and fill the syringe with it. One of the advantages, besides uniformity and slowness of rate, possessed by this type of apparatus is that, owing to the powerful driving force and the rigidity of the connections, slight obstructions do not appreciably interfere with the flow as they do in devices for a similar purpose which depend upon a delicate adjustment of pressure relations.

After commencing the transfusion, five to ten minutes were allowed to elapse before withdrawing the first sample of blood, so as to allow equilibrium to be obtained. Further samples of blood were taken at intervals of fifteen to twenty minutes, and the blood-sugar determined by Maclean's method.

Rate of Removal of Glucose from the Blood in the Well-fed Animal.

Cats were kept in the laboratory for a week before the experiment and fed on liver and lung, twenty animals in all being used. Various rates of injection between 0·1 and 0·2 gm. of glucose per kilo per hour were tried in order to

determine the average rate which would keep the blood-sugar level after ligation of the portal vessels. The average proved to be approximately 0·15 to 0·16 gm. per kilo per hour (Fig. 3). This figure probably represents the minimum rate at which the liver must supply glucose to the tissues in order to maintain the blood-sugar at the normal level, the animal being in the resting condition and no absorption of food taking place from the alimentary canal.

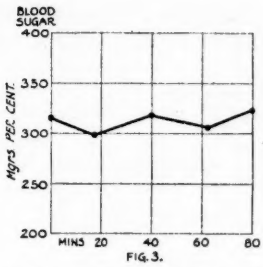


FIG. 3.

Same as Fig. 2, but with glucose transfusion, 0·15 gm. per kilo per hour.

EXPERIMENTS.

The Effect of Insulin.

In studying the influence of insulin upon the blood-sugar after the exclusion of the liver and during constant infusion of glucose, the blood-sugar was examined on three occasions at intervals of 15 to 20 minutes before injecting insulin, in order to determine whether the blood-sugar level remained approximately constant. After injecting the drug, the blood-sugar was again examined at intervals of 20 minutes. Those experiments in which the blood-sugar did not remain at a fairly uniform level during the period immediately preceding the injection were discarded. Frequently there was a drop during the first 15 minutes after the glucose transfusion was begun, particularly when the initial concentration of sugar in the blood was high, but in the second period the curve usually ran more level. The dose of insulin employed was 10 units per kilo, and Fig. 4 shows the fall in blood-sugar which follows the administration of the drug.

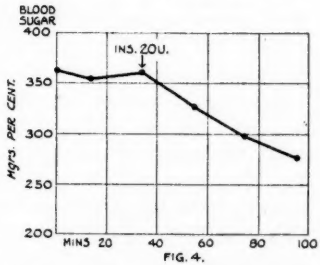


FIG. 4.

Same as Fig. 3, showing fall in blood-sugar following injection of insulin, 20 units intravenously.

Measurement of Increased Rate of Removal under Insulin.

The method employed was to increase the strength of the solution of glucose injected during the period with insulin, no alteration being made in the volume. This was accomplished by fixing to the injection apparatus a second syringe, containing the stronger solution, connected with the opposite jugular. It was brought into action either by reversing the machine or by merely putting it in the place of the first syringe.

When the rate at which glucose was supplied was 75 per cent. greater after the administration of insulin, the curve of blood-sugar showed only a slight drop or remained level. On the other hand, an increase of 100 per cent. was invariably followed by a gradual rise, even when the blood-sugar had been previously falling (Fig. 5). The acceleration must therefore be between 75 and 100 per cent., so that the maximum rate of removal

of glucose under these conditions must be somewhat less than 0·3 gm. per kilo per hour. This was less than might have been expected, but possibly a greater increase in the rate of glucose utilization under the influence of insulin may occur in the case of a muscle in activity. Nevertheless Dale and Burn found that when the isolated heart was perfused with diluted blood, glucose disappeared from the perfusion fluid at a rate 74 per cent. greater when insulin was added than without. When it was perfused with Locke's solution, however, the addition of insulin caused an increase of 100 to 150 per cent.

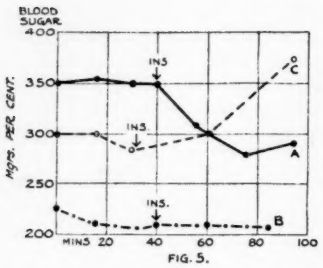


FIG. 5.

Same as Fig. 4, but increasing the rate of glucose transfusion after the administration of insulin: A, increased 70 per cent.; B, increased 75 per cent.; C, increased 100 per cent.

The importance of keeping the volume of the injected fluid small and constant in the experiment upon the decerebrated cat must be borne in mind, as the injection of large and increasing volumes has a pronounced influence upon the apparent rate at which glucose disappears from the circulation. Thus, after injecting 10 c.c. of Bayliss's gum solution per kilo at the beginning of the experiment, the rate of injection necessary to balance the fall without insulin may be over 0·20 gm. per kilo per hour. This may perhaps be accounted for by the improved circulation and the widening of the capillary bed, and therefore of the area over which diffusion of glucose into the tissues may occur. The influence of dilution must also be taken into account.

Effect of Leaving the Liver and Pancreas Intact.

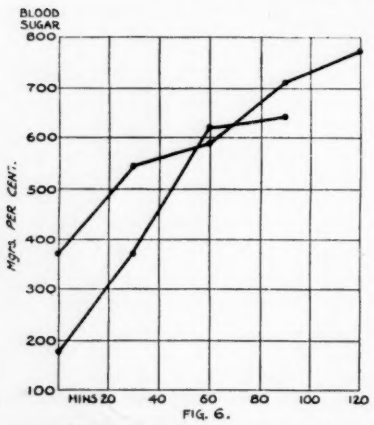


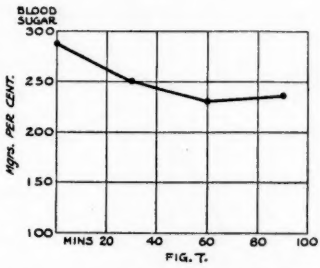
FIG. 6.

Two experiments on cats, showing hyperglycæmia following decerebration when splanchnics remain intact.

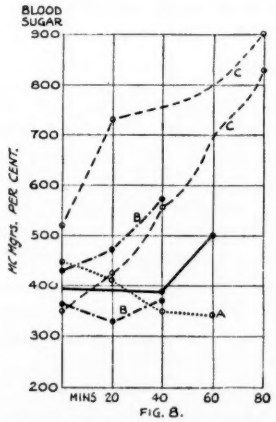
Since Woodyatt showed that in the rabbit, dog and man the maximum rate at which glucose could be injected into the intact animal over long periods without causing glycosuria was on the average 0·85 gm. per kilo per hour, this has been regarded as the rate at which glucose utilization takes place. The maximum rate at which the tissues, exclusive of the liver, use up glucose after insulin under the conditions above described is somewhat less than half this figure, and this indicates that the liver must be responsible for the disappearance of about half the glucose supplied. It therefore seemed of interest to study the effect of leaving the liver intact, while glucose was administered as in previous experiments.

In the decerebrate preparations a

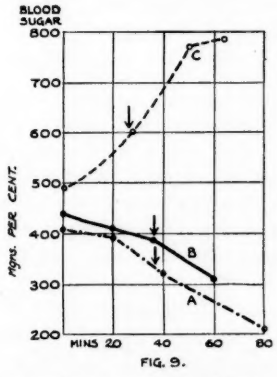
marked and increasing hyperglycæmia occurs owing to nervous impulses through the splanchnics causing glycogenolysis in the liver (Fig. 6). When the splanchnics are cut, however, the blood-sugar tends to fall a little (Fig. 7), while the blood-pressure is also lowered. After decerebration, therefore, both the splanchnics were cut, and after ligating the renal vessels the abdomen was closed, leaving the viscera intact. Various rates of glucose transfusion were tried in order to find how much could be given without causing a rise in the blood-sugar. The requisite quantity was found to be between 0·5 and 0·7 gm. per kilo per hour in animals fed in the manner already described (Fig. 8). The average, 0·6 gm. per kilo per hour, is, therefore, about four times the rate of removal in the other tissues without insulin, and double the maximum rate of removal in these tissues under insulin, or somewhat less than Woodyatt's constant. That the figures should fall short of Woodyatt's constant is not surprising, considering the unfavourable conditions, the lowered



Decerebrate cat; both splanchnics cut.



Decerebrate cat; both splanchnics cut, liver intact. Glucose transfusion at various rates: A, 0·5 gm. per kilo per hour; B, 0·7 gm. per kilo per hour; C, 0·8 gm. per kilo per hour.



Decerebrate cat; both splanchnics cut, liver intact. Glucose transfusion at various rates. Insulin, 20 units, at arrow. A, 0·15 gm. glucose per kilo per hour; B, 0·5 gm. glucose per kilo per hour; C, 0·8 gm. glucose per kilo per hour.

blood-pressure and an inevitable amount of shock. It may be noted that when Bayliss's gum solution, 10 c.c. per kilo, was injected at the beginning of the experiment, the glucose removal in the tissues was about 34 per cent. greater than without. An increase of 34 per cent. in the rate of removal by the liver

and tissues combined would bring the figure up to 0·8 to 0·9. When insulin, 10 units per kilo, was injected during glucose transfusion at the rate of 0·5 to 0·6 gm. per kilo per hour, a slight fall was noticed. With greater rates of injection the blood-sugar rose in spite of the extra dose of insulin (Fig. 9).

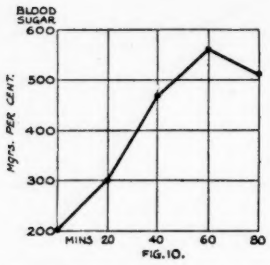


FIG. 10.

Decerebrate cat, liver intact, splanchnics intact. Glucose transfusion at 0·6 gm. per kilo per hour.

The fact that the injection of large quantities of insulin produced a relatively insignificant effect is probably due to there being already a considerable supply of insulin in the blood since the pancreas was intact. Further, any increased removal by the muscles if of the order previously found would be insignificant as compared with the influence of storage in the liver. That the increased removal of sugar when the liver is included is due to storage is, however, only a matter of conjecture, and does not in any way follow from the experiments. The continuous supply of glucose, keeping the blood-sugar at a high level, and the cutting of the splanchnics would nevertheless seem to provide conditions known to be favourable to glycogen formation in that organ. When the splanchnics are left intact the curve of blood-sugar rises rapidly when glucose is injected at the rate which keeps the blood-sugar about level when they are cut (Fig. 10).

Effect of Anæsthetics.

That anæsthetics produce hyperglycæmia through increasing the mobilization of glycogen in the liver is well known; but their effect upon the utilization of glucose in the tissues does not seem to have received attention. It therefore appeared desirable to make some observations upon this point, by employing the technique already described. In the first place it may be stated that the curves obtained after ligating the portal vessels under morphine and chloretone in the dog, urethane or ether in the rabbit, are comparable to those in the same animals after simple destruction of the brain. The cat, however, was selected for the study of the influence of ether and chloroform.

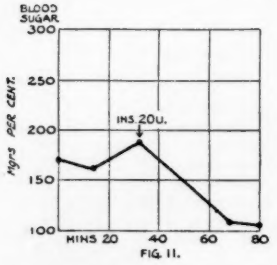


FIG. 11.

Evicerated cat, portal vessels ligated. Glucose transfusion 0·15 gm. per kilo per hour; ether anæsthesia throughout. Showing fall in blood-sugar after insulin, 20 units.

Ether and chloroform throughout.—Under ether or chloroform anæsthesia, without decerebration, the rate of injection of glucose necessary to keep the blood-sugar level after ligating the portal vessels was almost the same as that in the decerebrate preparation, namely about 0·15 grm. per kilo per hour, though there was perhaps a slightly greater tendency for the curve to rise, especially under chloroform anæsthesia. The rate of removal of sugar under anæsthesia is therefore comparable with that which occurs in the un-anæsthetized decerebrate animal.

Ether and chloroform with insulin.—Insulin produced the usual fall in the blood-sugar when administered after a preliminary control period, the animal having been under the influence of the anæsthetic for about an hour altogether before the dose of insulin was given (Figs. 11 and 12). When the rate of transfusion of glucose was increased by 75 per cent. during the period with insulin the blood-sugar fell slightly, showing that the increased utilization of sugar was comparable to that which occurs without anæsthetics. In an experiment with chloroform (Fig. 13) insulin was administered after 75 minutes of chloroform anæsthesia, which included the time taken up by the operation and the control period. During the latter the blood-sugar remained level, while a slight fall occurred after 20 units of insulin and an increase of 75 per cent. in the rate of glucose injection. The action of insulin would therefore appear not to be appreciably interfered with by the anæsthetic.

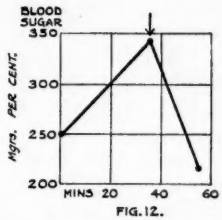


FIG. 12.

Same as Fig. 11, but with chloroform anæsthesia.

Ether in decerebrate preparation.—When ether was administered, after two preliminary periods without it, to the decerebrate animal with liver out and glucose transfusion at the usual rate to keep the blood-sugar level, no alteration was observed in the direction of the curve of blood-sugar, showing that no change in the rate of glucose removal occurred (Fig. 14). The

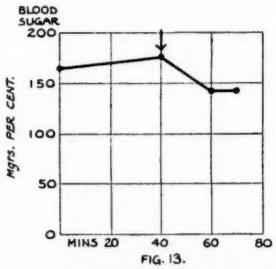


FIG. 13.

Chloroform anæsthesia throughout. Portal vessels ligated. Glucose 0·15 gm. per kilo per hour before insulin and 0·26 after insulin, administered at arrow.

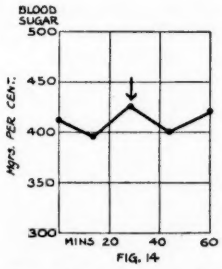


FIG. 14

Decerebrate cat, portal vessels ligated. Glucose 0·15 gm. per kilo per hour. Ether administration after arrow.

concentration of ether vapour employed was the usual ether used for general anæsthesia, obtained by mixing equal quantities of pure air and air saturated with ether vapour. This was administered through the artificial respiration apparatus.

Great difficulties were encountered in attempts to perform an analogous experiment with chloroform, since the dividing line between an anæsthetic and a toxic dose is small, and the animal's reactions were not available as a guide to the depth of the anæsthesia. No satisfactory result was obtained.

Ether with insulin.—After a control period without anæsthetics ether was administered as in the preceding experiments, and a dose of insulin given, when the usual fall in blood-sugar followed (Fig. 15). It has been shown by Banting, Best, Collip, Macleod and Noble that the hyperglycæmia caused by ether can be prevented by insulin. In view of the above findings one of the factors bringing about this result must be the increased removal of sugar in the tissues. Whether the glycogenolysis due to anæsthetics be inhibited by insulin as in the case of that due to adrenalin (Macleod and Noble) requires investigation.

Since the removal of sugar in the muscles under anæsthesia does not appear to be diminished beyond what might be expected to result from the absence of actual contraction, the glycosuria (and perhaps the acetonuria) which sometimes occurs after anæsthesia must probably be laid to the account of some poisonous action upon other organs controlling carbohydrate metabolism, especially the liver, and possibly the pancreas. It may be noted that although anæsthetics do not, apparently, alter the rate at which glucose is removed from the blood by the tissues, it does not follow that there is no interference with the oxidation of glucose in the tissues. The two processes may depend upon different mechanisms.

Effect of Pituitrin.

Burn has demonstrated an antagonism between pituitrin and insulin in the intact animal, pituitrin inhibiting the fall in blood-sugar due to insulin, or causing a return to the normal level after insulin hypoglycæmia. It seemed

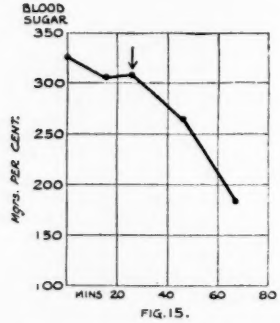


FIG. 15.

Same as Fig. 14. Ether + insulin (20 units) after arrow.

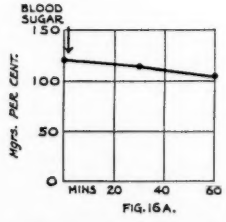


FIG. 16A.

Cat, intact animal. Pituitary extract 40 mgm. + insulin 10 units subcutaneously.

of interest to find out whether the antagonism would be exhibited in relation to the direct action of insulin upon the removal of sugar by the muscles. Cats were fed in the usual way, and to the intact animal 10 units of insulin + 3–4 c.c. of a saline extract obtained from 40 mgm. of an active dried extract of posterior lobe of pituitary gland were injected subcutaneously. Blood was

withdrawn from a superficial vein in the leg at intervals for blood-sugar examination. These doses were found to be antagonistic, little, if any, fall taking place in the blood-sugar at the end of an hour (Fig. 16A). A week later the same animals were decerebrated, and dealt with according to the usual technique, the liver being cut off from the general circulation, and glucose run in to keep the blood-sugar level. After the preliminary control periods the equivalent of 40 mgm. of dried pituitary extract in 3–4 c.c. of saline were

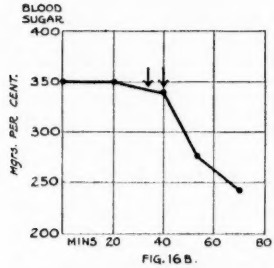


FIG. 16 B.

Cat (same as 16A), decerebrated; portal vessels ligated. Glucose 0·15 gm. per kilo per hour. Pituitary extract 40 mgm. at first arrow; insulin 10 units at second arrow.

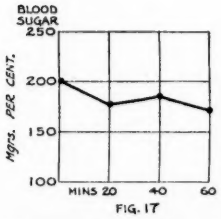


FIG. 17

Decerebrate cat. Portal vessels tied. Glucose transfusion 0·15 gm. per kilo per hour, pituitary extract 40 mgm.

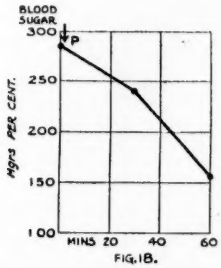


FIG. 18.

Same as Fig. 17, without glucose transfusion.

injected, and 10 minutes afterwards blood was withdrawn for examination and 10 units of insulin were injected. The blood-sugar remained unchanged during the 10 minutes with pituitrin alone, but showed the usual fall after insulin (Fig. 16B). In other experiments pituitary extract alone was given, while glucose was transfused at the usual rate, but no influence could be detected upon the character of the curve (Fig. 17). Similarly, pituitrin did not prevent the fall in blood-sugar after simple ligation of the hepatic vessels in the decerebrate animal (Fig. 18).

CONCLUSIONS.

(1) The average rate at which the tissues remove glucose from the circulation in the decerebrate cat with the liver tied off is 0·15 gm. per kilo per hour.

(2) This rate is increased by 75 to 100 per cent. by insulin in doses of 10 units per kilo.

(3) When the liver is included and the splanchnics are cut, the rate of removal averages 0·6 gm. per kilo per hour.

(4) The rate at which the tissues remove glucose from the circulation when the liver is excluded is about the same under anæsthesia as in the decerebrate preparation.

(5) The rate at which insulin accelerates the removal of glucose appears to be unchanged by the administration of anæsthetics.

(6) Doses of pituitrin which are sufficient to prevent the fall in blood-sugar under insulin in the intact animal fail to do so when the liver is excluded, and glucose is supplied at the rate necessary to keep the blood-sugar level during the control period.

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ON A GROWTH-PROMOTING FACTOR IN TUMOUR TISSUE.

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Received for publication December 4th, 1925.

It is a comparatively new conception that living cells require minute quantities of certain special substances derived from organic matter to maintain life and active proliferation. In cancer research this idea has not attracted much attention, for it has been usual to look upon the established malignant cell as abundantly supplied with all the food material that it requires. Yet the rate of growth of tumours in animals can be appreciably increased by inoculating a substance derived from the breaking-down products of tumour-cells.

It was shown in 1924 (Chambers and Scott) that if tumour tissue was rubbed down to a fine emulsion and washed many times with saline, and the solid residue allowed to autolyse at 42°C. in a solution saturated with toluene, a substance was produced which, if inoculated into animals, made them hypersensitive to tumour growth. The sensitized rats grew tumours which reached a volume of 34·4 c.c. in the same time that control tumours reached a volume 14·6 c.c. The extract producing this effect was a clear colourless fluid containing only a trace of proteins. The sensitized animals were apparently otherwise unaffected by the inoculation, yet the effect on the tumour-cells was undoubted, and was increasingly apparent as the tumours grew.

In the investigation described in the following paper it has been found that the stimulating substance is almost certainly a chemical product and not a living agent. It is formed under conditions in which bacteria do not multiply, and has been obtained by autolyzing tumour tissue in a solution of N/20 HCl. It withstands a temperature of 100° C. for ten minutes. It has been found in "anaërobic cultures" of Jensen's rat sarcoma grown under the conditions described by Gye (1925) for cultivating his "cancer virus." This chemical body has been inoculated into animals, and a test inoculation given a week later on the opposite side of the body. The tumour used was degenerate, and failed to grow in a third of the normal animals. Large tumours formed in all but one of the sensitized animals at the site where the degenerate cells had been inserted.

The research with which this paper is concerned has been in progress some six or seven years, and its main object has been to study the problems affecting tumour growth *in vivo*, especially the influence of the absorption of tumour tissue on the production of immunity. It has been known for more than fifteen years that two apparently opposite biological effects may result from the absorption of tumour material. An animal may become effectively protected against tumour growth, or it may become sensitized so that a tumour grows in it more rapidly than in a normal animal.

In a previous paper (1924) the writers have put forward the hypothesis that both immunity and stimulation are due to the absorption of products of the dead tumour-cells, and that these products are formed as a result of the actions of intracellular enzymes on the nuclear structures. To explain the two different effects, it was suggested that two bodies might be formed, and which of these was absorbed depended upon the varying conditions around the site of inoculation.

Further experiments support this hypothesis that both immunity and stimulation are due to changes in the inoculated material brought about by the action of intracellular enzymes, for if the action of these enzymes is prevented by shaking the tumour material with normal serum at 37°C. neither effect is produced, although a relatively large amount of tumour tissue is absorbed, and although the original structure has not, as far as we know, been altered.

The experiments which have been done during the last two years have been chiefly concerned with the conditions under which the substance producing stimulation of growth is formed.

TECHNIQUE.

The strain of tumour which has been used throughout this work, viz. Jensen's rat sarcoma (J.R.S.) has been the subject of many criticisms. These have been chiefly: (1) that the results are liable to great variation owing to the concomitant immunity that this tumour produces, and (2) that it does not cause metastases, and is therefore not comparable with the commoner types of malignant disease.

Jensen's rat sarcoma has been in continuous propagation in these laboratories for the last fifteen years. Its characters are therefore well defined: they have not changed since it was first introduced. It grows rapidly in a large percentage of rats, and we have found (1924) that if meticulous care is used in choosing non-degenerate material for inoculation, the control animals give fairly comparable results in successive experimental series. If slightly degenerate tumour is used for inoculation the percentage of takes is reduced and the tumours grow more slowly, but even so when groups of animals are inoculated from the same sample of tumour material they give comparable results. This strain of tumour is, in fact, the most convenient we have with which to work.

It is true that metastases do not occur often, but this is probably because the experiments are usually over in about four weeks. In animals which bear progressive tumours for several months metastases are common.

As tumour-cells can be grown outside the body without much difficulty,

the methods of *in vitro* research would at first sight seem to be particularly desirable for studying the factors affecting growth. By this means all the extraneous influences which are due to the general response of animals are eliminated. On the other hand, the technique of *in vitro* cultures includes many variables which are difficult to avoid; it also unavoidably introduces the substance which we have set out to test and which is formed during autolysis. It is well recognized that autolytic changes take place in all *in vitro* cultures of animal cells, and that they alter the composition of the medium. Autolysis begins as soon as the cultures are started, and the rate of autolysis is influenced by many factors created by the technique, such as the serum content of the medium, the relative quantity of fluid to the number of cells present and the pH. The conditions affecting growth are, in fact, constantly changing. For these reasons, the investigation with which this paper is concerned has been done almost entirely on the growth of tumour-cells *in vivo*, though the technique used has the disadvantage that it is laborious and slow, and requires large numbers of animals. The work has involved measuring and following the rate of growth of over 2000 tumours. The details of the technique have been discussed elsewhere (1924) and need not be repeated again here.

EXPERIMENTAL WORK.

The soluble products obtained from tumour-cells (J.R.S.) after varying times of autolysis in Drew's fluid have been tested for their effect on tumour growth, and it has been found that the substance causing stimulation appears soon after the onset of autolysis and persists for several days. It has not been found in extracts of fresh tumour tissue. Factors which favour autolysis, such as mechanical disintegration of the cells, washing them with so-called physiological fluids and increasing the temperature also favour the production of this body. Toluene does not prevent its formation, but when chloroform was substituted it was not detected. (This last experiment has not yet been repeated.)

The Effect of Fresh Serum.

Experiment 1.—Experiments were made adding minced J.R.S. to fresh sheep's serum in varying dilutions, viz. 1 volume of tumour to 50 volumes of serum, 1 to 12·5 and 1 to 3, 2 per cent. toluene being added to prevent bacterial growth. The mixtures were kept in an incubator at 37° C. for varying times of 2, 3, 4, 5 and 7 days, and constantly shaken. The fluids were then centrifugalized and inoculated into different series of rats (1 c.c. into each rat and 20 animals in each series); a second treatment was given with similarly prepared fluid seven days later. After another week each series of experimental animals and 30 controls were given a test inoculation. In all these experiments no effect could be detected after treatment with the serum extract unless autolytic changes had occurred in the tumour tissue.

Autolysis is delayed or entirely prevented by shaking minced tumour with fresh serum. The effect depends upon the proportion of tissue to serum, and upon maintaining intimate contact between the fluid and the cells. When the quantity of serum is reduced below the amount which prevents enzyme action *i. e.* below 1 in 5 in the case of J.R.S. and sheep's serum, autolysis

takes place, but at first relatively slowly. When the serum is still further reduced enzymes are rapidly liberated and the serum extract develops marked proteolytic properties.

Stimulation of growth was obtained with serum after 3 days' incubation at 37° C. with tumour tissue in a dilution of 1 in 3, though not after 24 hours. A marked stimulation effect was obtained after 8 hours' incubation when the quantity of serum was still further reduced. The fluid now caused an acute œdema at the site of inoculation and extensive ulceration of the overlying skin.

In the experiment with 1 in 12·5 dilution, J.R.S. was used which had been given a "lethal dose" of X-rays (Chambers, Scott and Russ, 1922). After 5 days' incubation in sheep's serum at 37° C., the tumour-cells still retained their staining properties and showed no sign of autolysis. The mixture was then centrifugalized and 20 rats inoculated with the solid deposit (0·15 c.c. into each). Another 20 rats were inoculated with the fluid (1 c.c. per rat), and the dose repeated a week later. Test inoculations were given a week after the last treatment to all the 40 experimental animals and 30 controls. No difference could be detected in the size of the tumours in the two groups. It has already been shown, and repeatedly confirmed, that if fresh X-rayed J.R.S. is inoculated into rats a high degree of immunity is produced. Stopping the action of the intra-cellular enzymes apparently prevents both immunity and stimulation.

The Effect of Autolysis in N/20 HCl.

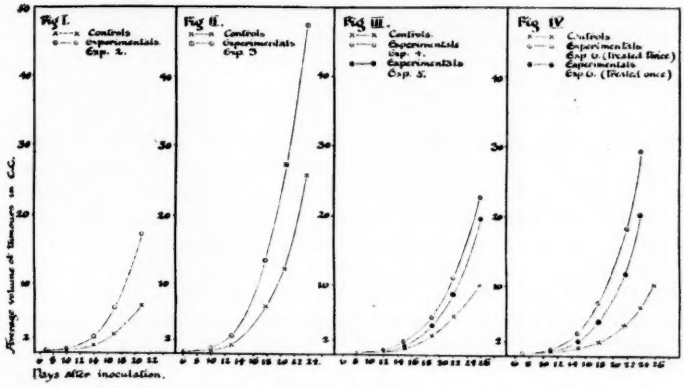
It has been shown by Dernby (1918) that tissues contain two kinds of proteolytic enzymes, one like pepsin carrying the digestion to the peptone stage and acting in an optimum pH of 3·5, and the other like trypsin and erepsin acting in an optimum pH of 7·8, and carrying the digestion further and inhibited in acid surroundings.

Experiment 2.—An experiment was made in which 12 c.c. of minced J.R.S. were rubbed down in a mortar to a fine emulsion and washed many times with normal saline, and then with a solution of N/20 HCl in normal saline. The solid residue was then taken and made up to a volume of 40 c.c. with fresh N/20 HCl solution. The mixture was incubated at 37° C. for 3 hours and then neutralized with caustic soda N/1. The fluid, when centrifugalized, was quite clear and colourless, but became slightly turbid on boiling, 18 rats were inoculated on the left side with 1 c.c. each of this clear unboiled fluid; no local reaction occurred: 7 days later all the experimental animals and 24 controls were given a test inoculation on the right side. Table I and Fig. 1 show the results obtained. It will be seen that after 21 days the average volume of the tumours in the experimental animals reached 17·3 c.c., compared with 6·7 c.c. in the controls.

TABLE I.

		Number of rats.	Days after inoculation. 7.	10.	14.	17.	21.
Average volume of tumours in c.c.	Controls . .	24	0·12	0·25	1·98	2·60	6·72
	Experimentals .	18	0·15	0·42	2·40	6·45	17·8

In this experimental series the test inoculations were given with tumour material which had undergone some degeneration, and in consequence in 8 of the control animals the tumours failed to grow. Only one tumour failed to grow in the sensitized animals, though great care had been taken to treat both groups with comparable material; an animal of each group was inoculated alternately from the same sample of tumour material. Apparently the sensitizing dose had not only made the tumours grow larger, but had made cells grow which would not grow in normal rats.



The Effect of Heat.

Experiment 3.—Experiment 2 was repeated. Some of the clear fluid obtained after neutralization was heated in a test-tube to 100° C. for 10 minutes; it was now faintly turbid. Twenty normal rats were inoculated on the left sides with this fluid (1 c.c. per rat). Eight days later all the experimental animals and 29 controls were given a test inoculation on the right sides. The results are shown in Table II and Fig. 2. It will be seen that the tumours in the experimental animals reached a volume of 47·4 c.c. in the same time that the control tumours reached 25·6. Heating the fluid to 100° C. had not destroyed its stimulating properties.

TABLE II.

		Number of rats.	Days after inoculation. 6.	10.	13.	18.	21.	24.
Average volume of tumours in c.c.	Controls . .	29	0·11	0·44	1·47	7·58	13·3	25·6
	Experimentals .	20	0·12	0·56	2·50	13·6	27·1	47·4

The Effect of Anaërobic Conditions.

Experiment 4.—Culture tubes were prepared containing 5 c.c. each of Hartley's broth to which 0·2 per cent. KCl had been added (Gye, 1925). 1 c.c. of fresh rat's serum was put into each tube and also a piece of fresh Jensen's

rat sarcoma. The tubes were incubated at 37° C. in a McIntosh and Fildes jar under strictly anaërobic conditions for six days. The jar was then cooled before being opened, and the fluid from four of the tubes which had remained free from bacterial contamination was pooled. Twelve rats were inoculated with 1 c.c. of this fluid on the left sides. A week later all the experimental animals and 30 controls were given test inoculations on the right sides. The results are shown in Table III and Fig. 3. It will be seen that the tumours in the experimental animals reached a volume of 22·6 c.c. compared with 9·7 c.c. in the controls.

Experiment 5.—18 c.c. of minced J.R.S. were rubbed down in a mortar and washed many times with normal saline. The solid residue was then put into a flask and made up to 180 c.c. with normal saline and toluene added. The air in the flask was then exhausted and replaced by hydrogen and the flask sealed. It was incubated at 37° C. for 18 hours and then cooled before being opened. The fluid was centrifugalized and 1 c.c. inoculated into the left sides of 10 normal rats. A week later the experimental animals together with those of Experiment 4 were given a test inoculation. The results are shown in Table III and Fig. 3. It will be seen that the tumours in the experimental animals reached a volume of 19·7 c.c. in the same time that the control tumours reached a volume of 9·7 c.c.

Although the Experiments 4 and 5 were done on a comparatively small number of animals, they both show that the stimulating body is formed under anaërobic conditions.

TABLE III.

		Number of rats.	Days after inoculation.					
			8.	12.	15.	19.	22.	26.
Average volume of tumours in c.c.	Controls . .	29	0·11	0·39	1·07	2·40	5·10	9·70
	Experimentals (Experiment 4.) .	12	0·12	0·51	1·97	5·32	10·9	22·6
	Experimentals (Experiment 5.) .	10	0·09	0·42	1·23	4·01	8·63	19·7

The Effect of "In-vitro" Cultivation.

Jensen's rat sarcoma can be grown very easily *in vitro* in Soyka flasks. A mixture consisting of 4 parts of serum and 1 part of distilled water is put into the flasks and coagulated at 100° C. in a steamer in such a position that a uniform layer of jelly is formed on one surface of the flask. The kind of serum is immaterial, but rat's serum gives a clearer jelly than either horse-, sheep- or ox-serum, and has been used for the experiments described here. Six or ten small pieces of fresh tumour are placed on the surface of the jelly by means of a platinum loop, and ½ to 1 c.c. of fresh rat's serum, which has not been exposed to air, is run into the flask with a sterile pipette. The flask is then quickly corked with a rubber cork. The surface of the jelly is flushed with serum and the flask incubated at 37° C. in a sloping position. No fresh serum is added, but each day the surface of the jelly is flushed by tilting the flask with the same serum which was first put in. Active growth takes place

in 100 per cent. of the pieces of tumour and continues for 5 days. Growth then practically ceases, but not because of any change in the medium, for if fresh pieces of tumour are added without adding fresh serum these grow as before for the next 5 days. This has been repeated for a third time. The growth forms branching processes into the jelly and spreads on the surface; pieces of tissue which measured originally 1 mm. across at the end of 5 days measured 5 to 8 mm. in diameter.

When tumour tissue (J.R.S.) which has been given a "lethal dose" of X-rays is incubated on the jelly, active growth apparently takes place for 5 days, and under low-power magnification the colonies are indistinguishable from those obtained with normal non-irradiated tumour.

Experiment 6.—Eight colonies of normal J.R.S grown in this way for 5 days were inoculated into 8 normal rats. Three tumours formed at the sites of inoculation.

Twenty-one colonies of X-rayed J.R.S grown for 5 days under the same conditions were inoculated into the left sides of 21 normal rats. Each colony with the surrounding jelly was removed with a small spatula and then inoculated. Thirteen of these rats were given a second treatment. No tumours developed at the sites of inoculation. A week after the last treatment all these 21 rats together with 30 controls received a test inoculation on the right sides. The results are given in Table IV, Fig. 4. It will be seen that in the experimental animals treated, once the volume of the tumours reached 20·2 c.c., in those treated twice 29·4 c.c., compared with 6·50 c.c. in the controls.

TABLE IV.

		Number of rats.	Days after inoculation.						
			7.	11.	15.	18.	22.	24.	29.
Average volume of tumours in c.c.	Controls	30	0·12	0·29	0·96	1·91	4·31	6·50	10·1
	Experimentals (Treated once.)	8	0·13	0·37	1·88	5·02	11·5	20·2	—
	Experimentals (Treated twice.)	13	0·16	0·52	3·10	7·77	18·3	29·4	—

The immunizing effect obtained with X-rayed J.R.S. is lost if the tumour is cultivated *in vitro* for 5 days prior to inoculation. Under these conditions the substance stimulating growth is obtained.

DISCUSSION.

It was first recorded by Bashford, Murray and Haaland (1908) that animals can acquire a condition of increased sensitiveness to tumour growth by treatment with mamma emulsions or emulsions of skinless embryos. Two years later Haaland (1910) showed that if mice were treated with carcinoma tissue or with mouse-embryo skin which had been mechanically disintegrated, they grew larger tumours than control animals; this was confirmed by Higuchi (1912).

The presence of substances in tissue juices which can accelerate growth has been recorded by many investigators working with *in vitro* cultures of

animal cells, notably by Carrel (1913, 1924) and Drew (1922, 1923). Carrel recorded (1913) that substances accelerating growth could be found in many types of tissues, embryo and lymphoid tissue being especially active. The addition of embryo extract to cultures of normal chicken fibroblasts increased their growth 2–40 times. If the extract was kept for 24 hours in the refrigerator the effect was more marked, but it deteriorated on keeping for a week, and the activity was lost on heating to 56° C.

The biological literature of the last ten years contains numerous references to the influence of cell products on the growth of living cells, and much evidence has been produced to show that these effects are not dependent on the nutritive value of the material.

The problems involved in this work are clearly closely related to the subject of vitamins. In this latter field most of the work which has been done (see 'Report on Accessory Food Factors, Special Report Series, Medical Research Council,' 1924) has been with living or dried foods or their extracts. Except in the case of yeast and bacteria, very little attention has been devoted to the vitamin content of autolytic products, although these must have been present to some extent in some of the substances tested. It is significant that vitamins are especially found in young and growing tissues.

In this investigation many more experiments have been done than those recorded here. The work has been much hampered by the fact that we are entirely ignorant of the chemical nature of the substance we have been testing, and we have had no other test for its presence than the effect on tumour growth.

SUMMARY.

A substance is produced from tumour-cells during autolysis which *in vivo* causes an appreciable increase in the rate of tumour growth.

The substance is formed under conditions which preclude its being a living agent. It is a fairly stable chemical body which is apparently derived from the nuclear structures.

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THE PREPARATION OF "PURIFIED" TOXIN FROM BLOOD-BOUILLON CULTURES OF *STREPTOCOCCUS SCARLATINÆ.*

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Received for publication December 3rd, 1925.

In the course of certain investigations regarding the toxin produced by strains of hæmolytic streptococci isolated from the throat in cases of scarlet fever, we have utilized a convenient method of obtaining toxin preparations for the Dick reaction, by which filtration of the culture can be omitted and a considerable amount of the inactive material derived from the added blood is eliminated.

The culture medium used was a standardized nutrient bouillon (pH 7·5) containing 5 per cent. defibrinated rabbit's blood (following the method of Ker, McCartney and McGarrity, 1925), and the cultures were incubated for 5 days. An abundant growth is obtained in this medium with a high content of the toxin responsible for the Dick reaction. Zingher (1924), Huntoon (1924) and others advocate the use of blood media in the preparation of the toxin, and we have also found that a medium enriched with blood gives a better yield of toxin (as judged by comparative skin reactions in known positive reactors) than one containing a corresponding amount of serum.

A toxin preparation, suitable after appropriate dilution for the Dick reaction, can be obtained in the usual way by centrifuging the culture, and then filtering the supernatant fluid through a Berkefeld filter. *Streptococcus scarlatinæ* growing in this medium produces almost complete lysis of the blood. The filtrates are deeply hæmoglobin-coloured, and contain the soluble products of hæmolysis in addition to the constituents of the blood-serum and the bouillon.

It has been found that if the unfiltered culture is exposed in flasks or tubes at 57° C. for one hour in a water-bath, an abundant brown flocculent precipitate forms, which can easily be separated by centrifuging, leaving a clear brownish-yellow supernatant fluid. This fluid contains apparently all the active toxic constituents of the culture. The culture itself undergoes sterilization as a result of the heating, and in this way, by taking advantage of the relative thermostability of the toxin, the toxin-containing portion of the culture can be readily separated from a considerable amount of the inactive constituents derived from the blood. At the same time filtration is rendered unnecessary. The precipitate by itself is apparently non-toxic.

The application of this method of obtaining toxin preparations suitable for the

Dick reaction has been carefully tested. A strain of *Streptococcus scarlatinæ* was used which was known to produce in rabbit's blood broth a toxin yielding a high percentage (95 per cent.) of positive Dick reactions in early scarlet fever cases, and a low proportion of positive reactions (4·7 per cent.) in cases from the 31st to the 35th days (Joe, 1925). The supernatant fluid after exposure of the culture at 57° C. (" 57°-soluble fraction ") was tested in a series of 50 cases in the Edinburgh City Fever Hospital by the Medical Superintendent, Dr. W. T. Benson, to whom we are indebted for carrying out the tests and recording the results. Simultaneous tests were also carried out in the same individuals with the usual filtrate preparation from the same culture. 0·2 c.c. of a 1-in-1000 dilution was the quantity used for the intradermal test. Control tests with the same preparations heated at 100° C. for one hour were also made in each series. This series of cases included patients with early scarlet fever and also convalescents. The results were practically identical with both the filtrate and the 57°-solution fraction. Each individual who showed a positive reaction with the former also reacted to the latter. A similar correspondence was noted in the case of negative reactors. Similar parallel tests were also carried out in 12 laboratory workers, 6 of whom showed equal positive reactions with both the filtrate and the 57°-soluble fraction.

The fraction precipitated by exposure at 57° C. was suspended in saline and made up to the original volume of the fluid from which it was derived. A 1-in-1000 dilution was tested according to the usual procedure for the Dick reaction in two laboratory workers who exhibited strongly positive reactions with the ordinary filtrate preparations of the toxin and with the 57°-soluble preparation. It was found to be quite inactive. In subsequent tests a higher concentration (made up to the volume of the original fluid and then diluted 1 in 400) of the suspended precipitate was used in persons who showed marked Dick reactions, with negative result.

Though it was not feasible to carry out quantitative tests with a series of dilutions in the cases used for the comparison of the different preparations, it was apparent that the 57°-soluble fraction of the culture represented for the most part the active toxic constituents.

The substances precipitated from the culture on heating for one hour at 57° C. have not been completely investigated, but appear to be derivatives of the lysed blood.

In this way, where blood-broth media are used in the preparation of the toxin, a considerable amount of inactive material derived from the added blood can be eliminated in a simple manner, and at the same time a sterile toxic product can be obtained without filtration. 0·5 per cent. phenol can then be added to the preparation for preservation, without interference with its properties. The toxin is apparently quite stable at 57° C. for one hour's exposure.

It was found that the active toxic constituents of the 57°-soluble fraction could be precipitated with 6 volumes of absolute alcohol. This precipitate, separated by centrifuging, was practically all re-soluble in saline, forming a yellowish solution, and the solution proved as active in the Dick reaction as an untreated filtrate from the original culture. This was ascertained in a series of 42 hospital cases, including early scarlet fever cases and

convalescents (tested by Dr. Benson). For purposes of testing, the saline solution was made up to the volume of the original fluid from which it was derived and a 1 in 1000 dilution was then prepared. In those cases in which a positive reaction occurred with the original filtrate, a similar result was also obtained with the alcohol precipitate from the 57°-soluble fraction. In one case this preparation gave a definite, though faint, positive reaction, while the filtrate preparation showed a doubtful reaction. It was noted also that in 5 other positive cases the reaction was a little brighter than that produced by the filtrate.

The alcohol used for precipitation was evaporated at 57° C., and the residue, which was of a brownish colour, was suspended in saline to the volume of the original fluid, then diluted 1 in 1000 and tested in a known positive reactor with negative result.

In this way a further purification of the toxin can be effected without apparent loss of its characteristic properties.

Henry and Lewis (1925) have advocated a method of precipitating out the toxin from cultures by means of alcohol. They use the condensation fluid of cultures on solid media containing blood and the precipitate is dissolved in saline. We have found, however, that if filtrates of blood bouillon cultures are treated with five to six volumes of absolute alcohol an abundant brownish precipitate forms, which is mostly insoluble in saline even after 5 minutes' contact with the alcohol. From this precipitate the toxic substances can be extracted with saline, but complete extraction can only be effected by repeated treatment with several changes of saline, *i. e.* as judged by the skin reactions obtained with a suspension of the insoluble residue in a known positive reactor. The first saline extract showed a slightly yellowish colour. After complete extraction the residue was inactive, while the saline solution in the appropriate dilution gave positive reactions in known reactors equivalent to those obtained with an untreated filtrate of the original culture. It seems possible that the insoluble part of the alcohol precipitate corresponds to the precipitate formed after heating of cultures at 57° C.

The alcohol precipitation method of Henry and Lewis cannot be applied to filtrates of blood-bouillon cultures in the same direct manner as in the case of the culture material used in their own work: the precipitate contains a large amount of insoluble material, and to remove completely the active soluble portion repeated extraction is necessary, though the first extraction with salt certainly removes the major portion of the toxic constituent.

The method we have described for separating out inactive substances by coagulation at 57° C., treating the 57°-soluble portion of the culture with 6 volumes of alcohol and re-dissolving the alcohol precipitate in saline, affords a convenient means of obtaining a relatively pure preparation of the toxin from blood-bouillon cultures.

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ON THE EFFECT OF METHYLENE-BLUE INJECTIONS ON THE BODY TEMPERATURE OF THE RAT.

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Received for publication October 31st, 1925.

THE physiological effects of the administration of methylene-blue have been studied by various workers, and one of the principal facts which have emerged from the evidence available is its capacity to produce a rise in body temperature.

Koskowski and Maigre (1921) report that the injection of 0·2 gm. in dogs causes paralysis of the vagus and the parasympathetic nerve-endings, which is associated with a rise in blood-pressure. It is suggested that the removal of the vagus control of the liver results in more glucose being set free in the circulation and oxidized in the tissues. It is found that if the liver be excluded no hyperthermia is produced, and this is also true if the animal be curarized, so that it is concluded that the seat of the combustion of sugar is the muscles. A diminution of the inhibitory action of the vagus is also reported by Heymans (1922[1]), and this author (1922[2]) also confirms the elevating effect on the body temperature. In dogs a rise in the rectal temperature was noted, reaching 44–45° C., following the injection of 0·04–0·06 gm. of methylene-blue per kilo body-weight, and the author attributes this to a stimulation, either direct or indirect, of cellular metabolism. Heymans (1922[3]) found, in addition, that the hyperthermia was accompanied by an increased elimination of carbon dioxide, and that the greater the hyperthermia the greater was the CO_2 output. The latter was found to precede the rise in temperature, which is considered to be due to hypermetabolism of carbon.

A considerable amount of interest attaches to this action of methylene-blue, and it was decided by the writer to carry out a series of observations on rats with a view to determining the action on these animals of injections comparable to those used in the case of dogs.

Five experiments were carried out and the protocols of these are now given. The temperatures are on the Fahrenheit scale.

Experiment I.—Dose 1 c.c. 0·5 per cent. Methylene-blue in Saline.

Rat No.	Weight (kg.).	Dose per kg.	Temperature. Before injection.	Minutes after injection. 10.	30.	60.	90.	120.	180.
17	0·120	0·041	100·3	100·5	98·6	99·8	99·8	100·4	100·2
12	0·162	0·031	100·0	99·1	98·6	98·6	100·0	99·2	98·4
15	0·152	0·033	100·2	99·5	95·2	96·8	96·4	98·2	97·4
9	0·165	0·030	99·6	97·8	96·8	97·2	97·6	99·0	98·2
Averages . .			100·0	99·2	97·3	98·1	98·5	99·2	98·8

Experiment II.—Dose 2 c.c. 0·5 per cent. Methylene-blue in Saline.

Rat No.	Weight (kg.).	Dose per kg.	Temperature. Before injection.	Minutes after injection. 10.	30.	60.	90.	120.	180.
4	0·127	0·080	99·8	99·6	98·4	99·0	100·0	98·8	99·6
7	0·132	0·076	100·4	100·4	100·4	100·6	99·8	100·2	99·0
5	0·127	0·080	100·0	100·6	98·8	100·4	101·0	100·2	100·8
18	0·130	0·077	98·8	99·2	97·4	99·0	100·0	98·6	99·0
Averages . .			99·8	99·9	98·8	99·8	100·2	99·5	99·6

Experiment III.—Dose 1·5 c.c. 1 per cent. Methylene-blue in Saline.

Rat No.	Weight (kg.).	Dose per. kg.	Temperature. Before injection.	Minutes after injection. 10.	20.	30.	60.	90.	180.
21	0·110	0·136	100·2	100·2	100·0	101·0	100·2	99·8	99·6
22	0·110	0·136	100·0	99·8	99·8	100·0	98·4	100·0	100·8
25	0·110	0·136	99·4	99·6	100·0	99·4	99·6	99·6	100·0
11	0·112	0·134	97·6	98·0	97·8	98·0	99·0	98·2	98·4
Averages . .			99·3	99·4	99·4	99·6	99·3	99·4	99·7

Experiment IV.—Dose 2 c.c. 1 per cent. Methylene-blue in Saline.

Rat No.	Weight (kg.).	Dose per kg.	Temperature. Before injection.	Minutes after injection. 10.	30.	60.	90.	120.	180.
13	0·120	0·170	100·6	100·4	100·2	100·4	101·0	100·4	100·2
29	0·165	0·121	101·2	100·8	99·6	100·0	100·4	100·0	100·8
3	0·118	0·170	100·8	100·8	101·2	100·0	100·4	101·0	100·8
6	0·115	0·174	99·4	100·2	99·4	99·8	99·8	100·0	99·6
Averages . .			100·5	100·6	100·1	100·1	100·4	100·4	100·4

Experiment V.—Dose 2 c.c. 2 per cent. Methylene-blue in Saline.

Rat No.	Weight (kg.).	Dose per. kg.	Temperature. Before injection.	Minutes after injection. 30.	60.	90.	120.	180.	360.
16	0·160	0·250	100·2	99·4	100·4	101·0	99·8	98·6	101·4
23	0·195	0·205	101·2	100·8	100·4	101·0	100·4	100·2	100·6
26	0·205	0·195	100·8	100·8	100·6	101·0	100·4	100·6	100·4
20	0·190	0·215	100·0	99·4	99·8	99·8	100·0	99·4	99·6
Averages . .			100·6	100·1	100·3	100·7	100·2	99·7	100·5

The figures given by these experiments indicate that no abnormal fluctuations in temperature occur as a result of the injection of methylene-blue in rats. The normal body temperature of the rats in the author's colony has been reported elsewhere as averaging 100·4° F., but, as is generally recognized, fluctuations of two or three degrees may take place under apparently the same conditions in these animals, and, as Donaldson (1924) points out, the mechanism for regulation of the body temperature in the rat is by no means efficient. It is consequently concluded that the variations seen in the experiments detailed above are no more than might be expected under normal conditions, and certainly no increases comparable to those seen in dogs were recorded, even although the amount of methylene-blue injected in some cases was four times as much as was sufficient to raise the rectal temperature in dogs to 111–113° F.

Whether this failure in rats to react to methylene-blue can be correlated with the inefficient temperature-regulating mechanism in these animals is at present a matter of speculation, and no explanation can at the moment be put forward.

In the meantime the figures alone are given. They definitely indicate that the reaction seen in dogs is not applicable to rats and for this reason are of interest. The work is being continued, and it is hoped that at a later date further information, upon which an explanation can be founded, may become available.

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